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14. ABSTRACT

β2-microglobulin (β2M) is a signaling and growth-promoting factor stimulating prostate cancer cell proliferation and progression. Blockade of the β2M signaling axis resulted in the inhibition of androgen receptor (AR) and its target gene, prostate-specific antigen (PSA), and the induction of programmed death of prostate cancer cells *in vitro* and *in vivo*. Also, we identified a new *cis*-acting element, sterol regulatory element-binding protein-1 (SREBP-1) binding site, within the 5'-flanking human AR promoter region and its binding transcription factor, SREBP-1, regulating AR transcription by anti-β2M monoclonal antibody in prostate cancer cells. Furthermore, we revealed the novel molecular mechanism by which SREBP-1 promotes prostate cancer growth and progression. Alteration of SREBP-1 expression leads to regulate AR expression, cell growth, migration and invasion in prostate cancer cells. SREBP-1 also showed to induce fatty acid and lipid formation in prostate cancer cells through increase of fatty acid synthase expression. Additionally, SREBP-1 induced oxidative stress and NADPH oxidase 5 (Nox5) expression in prostate cancer cells. In subcutaneous xenograft mouse models, SREBP-1 significantly increased LNCaP tumor growth and promoted prostate tumor castration-resistant progression. These findings provided a new concept to reveal the role of β2M and its related signaling pathways, including AR, SREBP-1, fat metabolism and oxidative stress, contribute to prostate cancer growth, survival and progression, and further provides a new potential target to prevent and treat prostate cancer malignancy by using anti-β2M monoclonal antibody.

15. SUBJECT TERMS

anti-β2-microglobulin monoclonal antibody; androgen receptor; prostate cancer; sterol regulatory element-binding protein-1.

Table of Contents

INTRODUCTION:

Prostate cancer progression from an androgen-dependent (AD) to an androgen-independent (AI) state is well recognized clinically as a fatal event. Androgen signaling mediated by the androgen receptor (AR), a ligand-activated transcription and survival factor, is known to play a key role regulating this lethal progression (1, 2). The central molecule of this project is β2-microglobulin (β2M). β2M is a non-glycosylated protein composed of 119 amino acid residues, and the mature (secreted) form contains 99 amino acid residues with a molecular mass of 11,800 Da (3, 4). β2M associates with the heavy chain of major histocompatibility complex class I (MHC I) on cell surfaces (5). This complex is essential for the presentation of protein antigens recognized by cytotoxic T lymphocytes (6) and serves as a major component of body's immune surveillance mechanism (7). We previously showed that β2M plays an unexpected role mediating prostate cancer osteomimicry, cell growth, survival and progression (8, 9), and AR expression. In this project, we evaluate the molecular mechanism of AR gene expression at the transcriptional level regulated by β2M during prostate cancer progression. We also focus on the β2M-mediated signaling and AR as a therapeutic target using a novel anti-β2M monoclonal antibody (β2M mAb) for the treatment of lethal prostate cancer malignancy. There are two specific aims proposed in this project: **Specific Aim 1:** To determine the molecular mechanism by which the β2M-mediated signaling regulates AR expression in prostate cancer cells. **Specific Aim 2:** To determine the anti-tumor efficacy of β2M Ab on prostate cancer cells *in vitro* and pre-established prostate tumors in mice *in vivo*.

BODY:

1) Blockade of β2M downregulated AR and PSA expression in human prostate cancer cells—We previously reported that β2M is a growth and signaling-promoting factor for human prostate cancer cells (8). Target β2M using a sequence specific β2M siRNA (8) or β2M Ab (10) greatly inhibited prostate tumor growth and induced cell programmed death via a caspase-9 cascade pathway *in vitro* and *in vivo*. To test if interrupting β2M from extracellular sources may also affect AR and PSA expression as well as cell growth of prostate cancer cells, we employed a new agent, anti-β2M monoclonal antibody (β2M mAb), to neutralize extracellular β2M and interrupt its downstream signaling. As shown in Fig. 1A and 1B, β2M mAb (0 to 10 μg/mL) significantly decreased both mRNA and protein levels of AR and PSA in LNCaP (AD) and C4-2B (AI) cells in a dose-dependent pattern determined by semi-quantitative RT-PCR and Western blot. Considered the specificity of β2M mAb inhibitory effect, purified β2M protein could rescue AR and PSA inhibition by β2M mAb

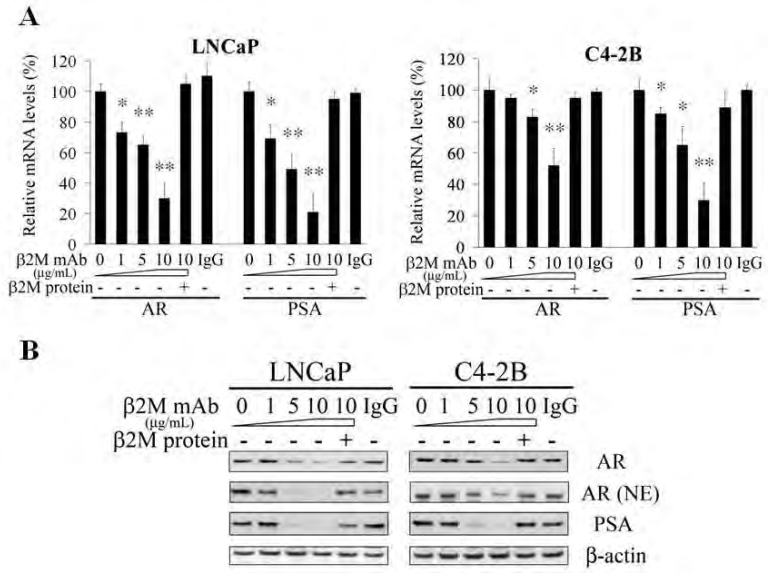

Fig. 1. 2M mAb decreased AR and PSA expression in prostate cancer cells. *A*,β2M mAb decreased AR and PSA mRNA expression in a dose-dependent manner (0 to 10 μg/mL) in both LNCaP (AD) and C4-2B (AI) prostate cancer cells determined by semi-quantitative RT-PCR. The inhibitory effect was restored by pre-incubation of β2M mAb with the same amounts of purified β2M protein. Isotype control IgG (10 μg/mL) did not significantly affect AR and PSA mRNA expression. The relative mRNA levels of AR and PSA, normalized by GAPDH mRNA, were measured by Gel Doc gel documentation software (Bio-Rad). The relative mRNA levels (%) were assigned as 100% in the absence of β2M mAb treatment. *, $P < 0.05$ and **, $P < 0.005$, significant differences from the β2M mAb-untreated group. Data represent the mean ± SD of independent triplicate experiments. *B,* β2M mAb also inhibited total AR, nuclear AR (NE) and PSA protein expression in a dose-dependent pattern (0 to 10 μg/mL) in LNCaP and C4-2B cells assayed by Western blot. The inhibitory effect was abrogated by pre-incubation of β2M mAb with β2M protein. Control IgG (10 μg/mL) did not change AR and PSA protein expression. β-actin was used as an internal loading control.

in prostate cancer cells. Control IgG did not affect AR and PSA expression. Not only decreased endogenous total AR protein, β2M mAb also showed to inhibit nuclear AR protein expression in LNCaP and C4-2B cells (Fig. 1B). These data demonstrated that antagonizing extracellular β2M by β2M mAb also reduced AR and PSA expression at transcriptional and translational levels in prostate cancer cells.

2) β2M mAb induced apoptotic death in prostate cancer cells—To determine the molecular mechanism by which β2M mAb inhibited the growth of prostate cancer cells, we first examined apopotic death in LNCaP and C4-2B cells, including sub-G_1 DNA content analysis and activation of caspase (11) and PARP expression. The results of flow cytometric analysis revealed that β2M mAb greatly increased sub-G_1 DNA contents in LNCaP (%sub G_1 = 82.49) and C4-2B (%sub G_1 = 79.45) cells compared to control IgG-treated LNCaP (%sub G_1 = 0.86) and C4-2B (%sub G_1 = 0.54) cells (Fig. 2A). Western blot analysis of caspases showed that cleaved caspase-9, caspase-3, and PARP, a downstream factor of caspases, were increased by exposing LNCaP and C4-2B cells to β2M mAb but not control IgG for a 48-h incubation (Fig. 2B). The induction of cleaved caspases and PARP was attenuated by pre-incubation of β2M mAb with purified β2M protein. In addition, cell death induced by β2M mAb was also confirmed at the level of light microscopy in LNCaP and C4-2B cells (Fig. 2C).

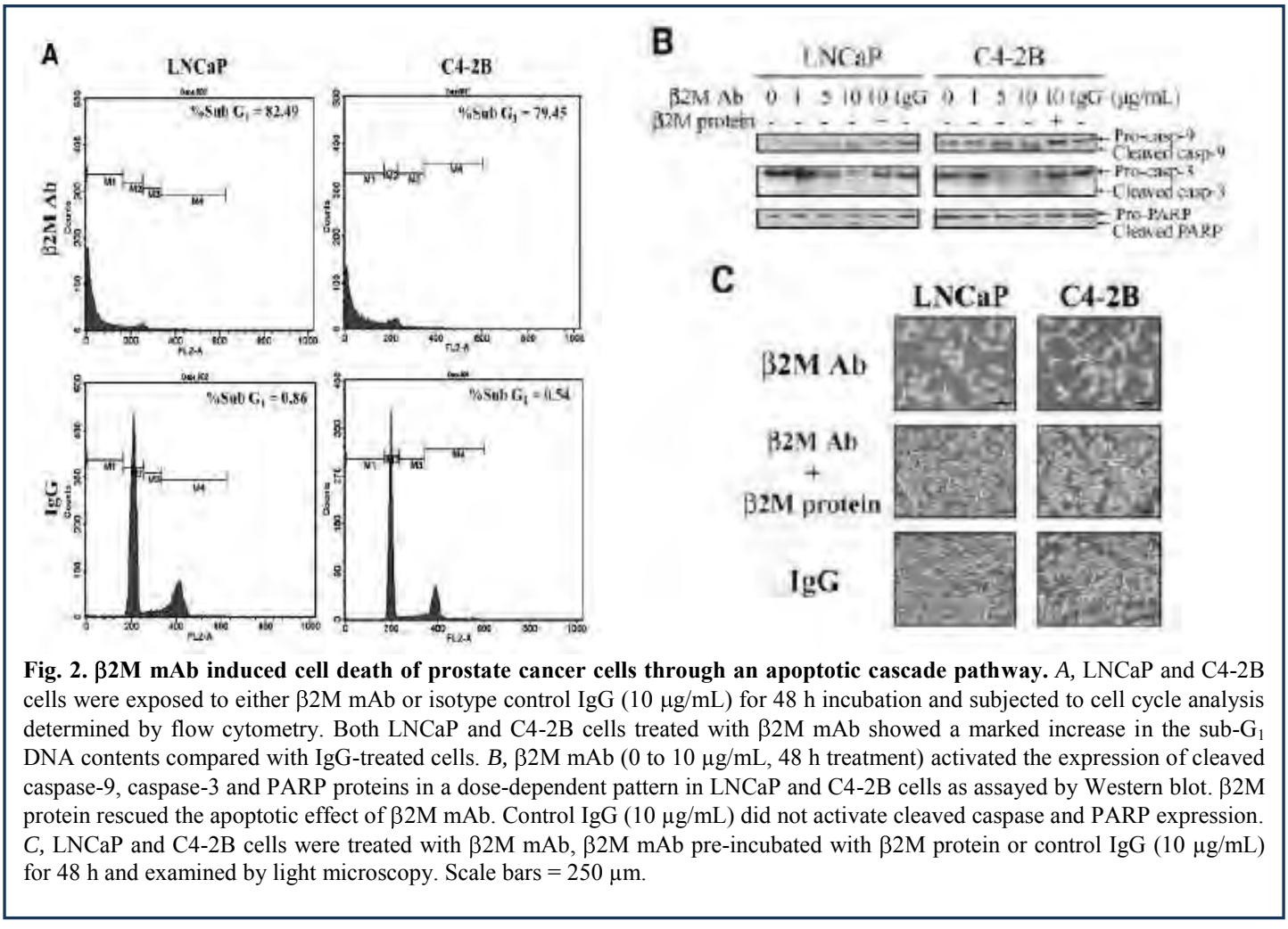

Fig. 2. β2M mAb induced cell death of prostate cancer cells through an apoptotic cascade pathway. *A,* LNCaP and C4-2B cells were exposed to either β2M mAb or isotype control IgG (10 μg/mL) for 48 h incubation and subjected to cell cycle analysis determined by flow cytometry. Both LNCaP and C4-2B cells treated with β2M mAb showed a marked increase in the sub-G_1 DNA contents compared with IgG-treated cells. *B,* β2M mAb (0 to 10 μg/mL, 48 h treatment) activated the expression of cleaved caspase-9, caspase-3 and PARP proteins in a dose-dependent pattern in LNCaP and C4-2B cells as assayed by Western blot. β2M protein rescued the apoptotic effect of β2M mAb. Control IgG (10 μg/mL) did not activate cleaved caspase and PARP expression. *C,* LNCaP and C4-2B cells were treated with β2M mAb, β2M mAb pre-incubated with β2M protein or control IgG (10 μg/mL) for 48 h and examined by light microscopy. Scale bars = 250 μm.

3) Sterol regulatory element-binding protein-1 binding site within the 5'-flanking promoter region of human AR gene is responsible for AR transcriptional activity regulated by β2M mAb—Subsequently, we sought to characterize the transcriptional mechanism of AR expression regulated by β2M mAb in prostate cancer cells. A luciferase reporter construct that contained the 5'-flanking human AR (hAR) promoter fragment (-5400 to +580) was transiently transfected into LNCaP and C4-2B cells. Consistent with previous RT-PCR and Western blot results (Fig. 1A and 1B), β2M mAb (0 to 10 μg/mL) significantly decreased hAR promoter luciferase activity in a concentration-dependent pattern (Fig. 3A). Purified β2M protein could restore the inhibition of hAR promoter reporter activity by β2M mAb as well. Isotype control IgG did not decrease hAR promoter luciferase activity in LNCaP and C4-2B cells. To further identify the responsible *cis*-acting element in the hAR promoter region, we conducted hAR promoter deletion study. Three deletion constructs of hAR promoter fragment (ΔA, ΔB and ΔC, Fig. 3B) were generated and confirmed the DNA sequence. After transfected into LNCaP and C4-2B cells, β2M mAb significantly inhibited the original hAR, ΔB (deletion of -1100 to -600) and ΔC (deletion of -1600 to -1100) promoter luciferase activities (Fig. 3B). However, β2M mAb did not affect the promoter luciferase activity of the ΔA construct (deletion of -600 to -40, Fig. 3B), and a

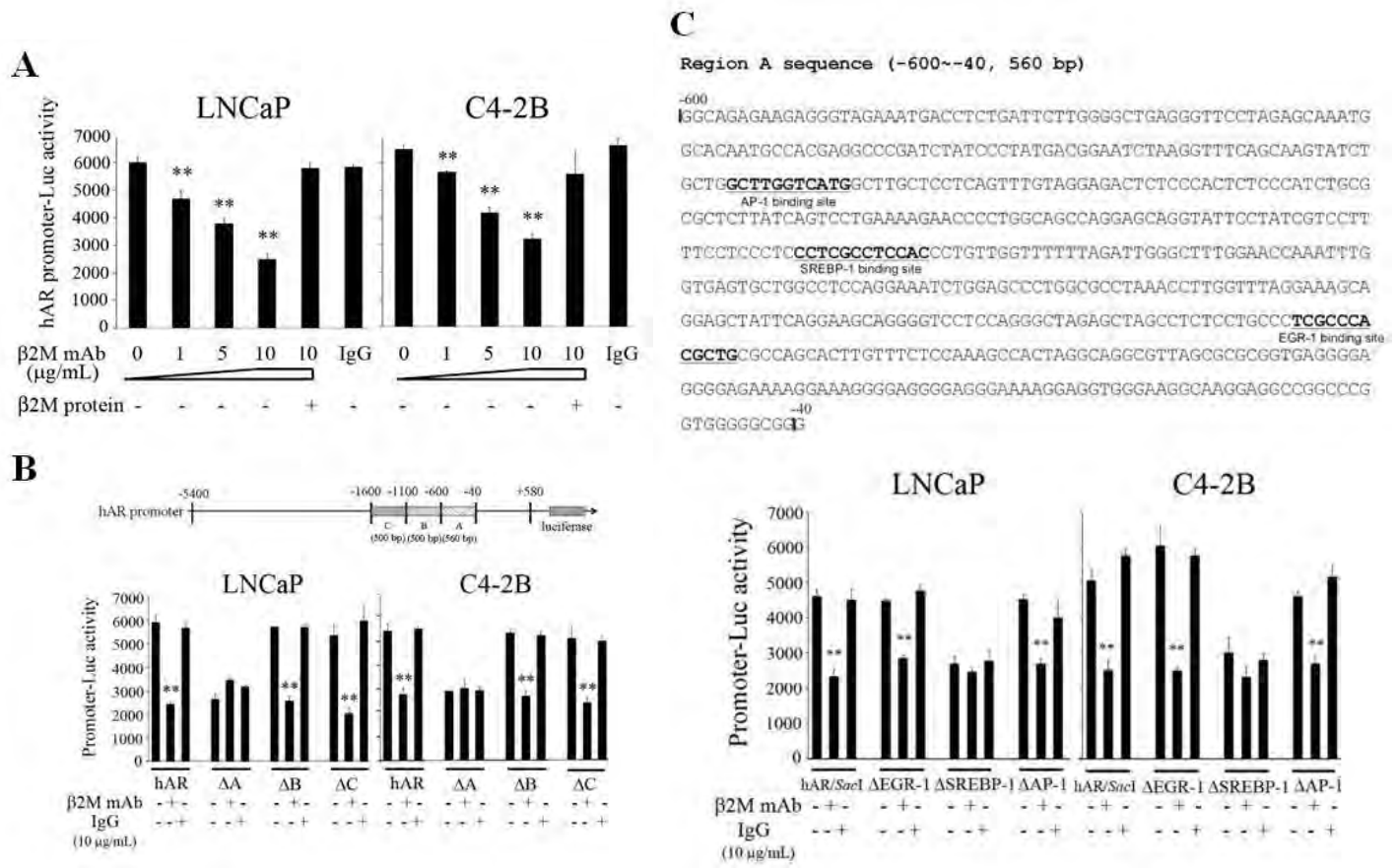

Fig. 3. Sterol regulatory element-binding protein-1 (SREBP-1) binding site within the 5'-flanking promoter region of human AR (hAR) gene is responsible for AR transcriptional activity mediated by β2M mAb. *A*, β2M mAb decreased hAR promoter (-5400 to +580) luciferase activity with a concentration-dependent pattern (0 to 10 μg/mL) in LNCaP and C4-2B cells. Purified β2M protein could restore the inhibitory effect of hAR promoter activity regulated by β2M mAb. Control IgG did not suppress hAR promoter reporter activity. *B*, Region A (-600 to -40) is responsible for hAR promoter luciferase activity mediated by β2M mAb in LNCaP and C4-2B cells. β2M mAb (10 μg/mL) significantly decreased the promoter luciferase activity of the deleted region B (ΔB, -1100 to -600) and C (ΔC, -1600 to -1100) in hAR promoter report constructs but did not affect the luciferase activity of the ΔA construct. Isotype control IgG (10 μg/mL) did not significantly change the promoter reporter activity of all deletion constructs. *C*, The DNA sequence of region A (-600 to -40, 560 bp) contains early growth response gene-1 (EGR-1) binding site (-181 to -170), sterol regulatory element-binding protein-1 (SREBP-1) binding site (-347 to -336) and activator protein-1 (AP-1) binding site (-475 to -465). Among the three deletion constructs (ΔEGR-1, ΔSREBP-1 and ΔAP-1 binding sites), the promoter luciferase activities of ΔEGR-1 and ΔAP-1 binding site constructs were significantly inhibited by β2M mAb in LNCaP and C4-2B cells. Only a slight drop of promoter luciferase activity was observed in a ΔSREBP-1 binding site construct while treated with β2M mAb in prostate cancer cells. Control IgG did not change the promoter reporter activities of these three deletion constructs. All promoter luciferase activity data were normalized by internal control β-galactosidase activity and expressed as the mean ± S.D. of three independent duplicate experiments. **, $P < 0.005$.

decrease in the basal promoter luciferase activity was observed only the ΔA construct in LNCaP and C4-2B cells. Control IgG did not significantly change the promoter activities of all these vector constructs. These results suggested that the region A within the hAR promoter fragment may contain the potential *cis*-acting element mediated AR transcriptional activity by β2M mAb. Because the original hAR promoter reporter vector contains approximate 6 kb (from -5400 to +580) in length, we further used a restriction enzyme, *Sac*I, to generate a shorter promoter luciferase construct, the hAR/*Sac*I vector (2 kb only, deletion of -4700 to -740), and tested this reporter vector activity in LNCaP and C4-2B cells exposed with β2M mAb or IgG. After luciferase activity assay, the basal promoter activity of this hAR/*Sac*I vector slightly decreased in LNCaP and C4-2B cells compared with the original hAR promoter (Fig. 3B and 3C), but no significant difference. It implied that the fragment, -4700 to -740, within the hAR promoter region, is not responsible for AR transcriptional activity regulated by β2M mAb in prostate cancer cells.

To further determine the β2M mAb-mediated *cis*-acting factor in the region A, based on the computer databank searched and analyzed, we predicted that three potential *cis*-acting elements in the region A may be responsible for AR transcription by β2M mAb, early growth response gene-1 (EGR-1) binding site (-181 to -170), sterol regulatory element-binding protein-1 (SREBP-1) binding site (-347 to -336) and activator protein-1 (AP-1) binding site (-475 to -465) (Fig. 3C). Subsequently, we generated three deletion constructs which are individually deleted these three transcription factor binding sites from the hAR/*Sac*I promoter luciferase vector and tested their reporter activity in prostate cancer cells. Among these three deletion constructs, the promoter luciferase activities of ΔEGR-1 and ΔAP-1 binding site constructs significantly inhibited by β2M mAb similar with the hAR/*Sac*I construct activity in LNCaP and C4-2B cells (Fig. 3C). Only a slight drop of promoter luciferase activity was observed in a ΔSREBP-1 binding site construct while treated with β2M mAb, and decreased the basal promoter luciferase activity of this construct (Fig. 3C). Control IgG did not significantly change the promoter reporter activities of all these deletion constructs. These hAR promoter deletion data, taken together, demonstrated that SREBP-1 binding site located within the 5'-flanking hAR promoter region is important for hAR promoter activity regulated by β2M mAb.

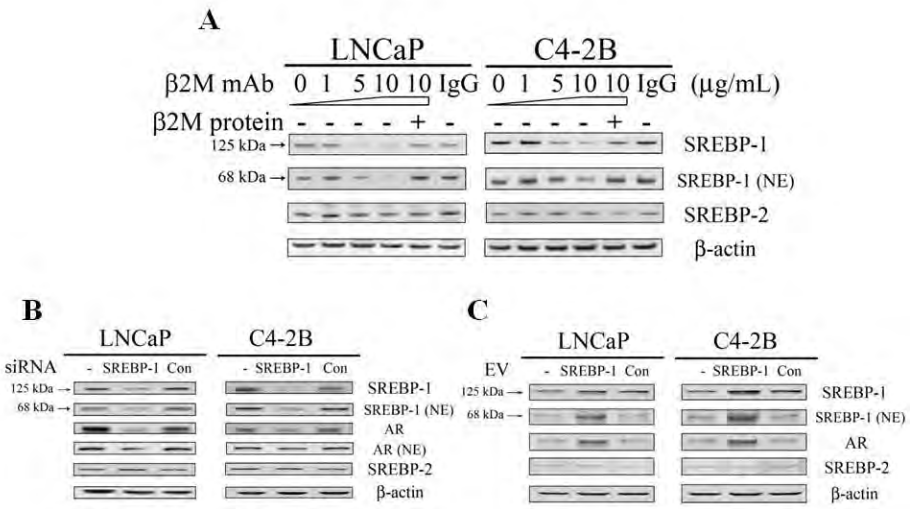

Fig. 4. The role of SREBP-1 in regulating endogenous AR expression in prostate cancer cells. *A*, β2M mAb decreased the expression of both precursor SREBP-1 (125 kDa) and mature nuclear SREBP-1 (68 kDa) in a concentration-dependent pattern (0 to 10 μg/mL) in LNCaP and C4-2B cells determined by Western blot. The inhibitory effect of endogenous SREBP-1 expression was restored by pre-incubation of β2M mAb with purified β2M protein. Isotype control IgG (10 μg/mL) did not affect SREBP-1 expression. β2M mAb did not change the expression of SREBP-2 which is an isoform of SREBP-1. β-actin was used as a loading control. NE, nuclear extracts. *B*, A sequence-specific siRNA of SREBP-1 decreased the expression of precursor and nuclear SREBP-1 proteins in LNCaP and C4-2B cells. Due to downregulation of SREBP-1 by SREBP-1 siRNA, AR and nuclear AR (NE) expression was also inhibited in LNCaP and C4-2B cells. For the specificity of this siRNA, SREBP-2 expression did not change by SERBP-1 siRNA. Control non-specific siRNA (Con) did not affect SREBP-1, AR and SREBP-2 expression. *C*, Overexpressing SREBP-1 by using a SREBP-1 expression vector (EV) increased the expression of precursor and nuclear SREBP-1 as well as endogenous AR protein in LNCaP and C4-2B cells. SREBP-1 expression vector did not affect SREBP-2 expression in prostate cancer cells. Con, control empty expression vector.

4) The role of SREBP-1 in regulating AR expression and cell viability in prostate cancer cells—We have shown that β2M mAb inhibited AR expression through the interaction of SREBP-1 and SREBP-1 biding site in prostate cancer cells. To examine whether β2M mAb also affected endogenous SREBP-1 expression, we performed Western blot to

evaluate precursor and nuclear SREBP-1 protein expression in LNCaP and C4-2B cells. As shown in Fig. 4A, β2M mAb (0 to 10 μg/mL) specifically inhibited expression of precursor (125 kDa) and mature nuclear (68 kDa) SREBP-1 proteins in a concentration-dependent manner but did not affect expression of SREBP-2, which is a SREBP-1 isoform. Purified β2M protein rescued the inhibitory effect of endogenous SREBP-1 expression by β2M mAb. Control IgG did not decrease SREBP-1 and SREBP-2 expression. Next, to investigate the role of SREBP-1 in regulating AR expression, we conducted the functional studies to knock-down and overexpress SREBP-1 in prostate cancer cells. A sequence-specific siRNA of SREBP-1 caused great decrease of both precursor and nuclear SREBP-1 proteins in LNCaP and C4-2B cells (Fig. 4B). Due to downregulation of SREBP-1, we also observed that expression of total AR and nuclear AR proteins was inhibited by SREBP-1 siRNA in LNCaP and C4-2B cells (Fig. 4B). SREBP-2 expression was not affected by SERBP-1 siRNA. Control non-specific siRNA did not inhibit expression of SREBP-1, SREBP-2 and AR. Conversely, we overexpressed SREBP-1 by using a SREBP-1 expression vector in prostate cancer cells. As an expectation, overexpressing SREBP-1 affected increase of precursor and nuclear SREBP-1 as well as AR protein expression, but not SREBP-2 in LNCaP and C4-2B cells (Fig. 4C).

5) SREBP-1 induces oxidative stress, Nox5 and catalase expression in prostate cancer cells. ROS and Nox (a ROS generator), have been reported to regulate cell proliferation, progression and metastasis, and radiation resistance of prostate cancer cells (12-14). Our cDNA microarray data showed that Nox5 was greatly up-regulated in SREBP-1 overexpresssing LNCaP cells compared to control cells (unpublished data). To further determine whether SREBP-1 induces prostate cancer cell proliferation through activation of Nox5 and ROS, we first examined expression of Nox5 in parental LNCaP, control Neo and SREBP-1 overexpresssing H1 and H2 (two highest SREBP-1 overexpresssing clones) cells. Consistent with the result of cDNA microarry, Nox5 protein increased in H1 and H2 compared to Neo and parental LNCaP cells (Fig. 5A). We also found that SREBP-1 increased p-Akt expression in prostate cancer cells (Fig. 5A), which is involved in prostate cancer cell proliferation, survival and progression (15). Next, we assayed ROS status (the levels of hydrogen peroxide and superoxide) in these SREBP-1 overexpresssing prostate cancer cells. SREBP-1 induced the levels of hydrogen peroxide in prostate cancer cells (Fig. 5B, the right panel). However, the

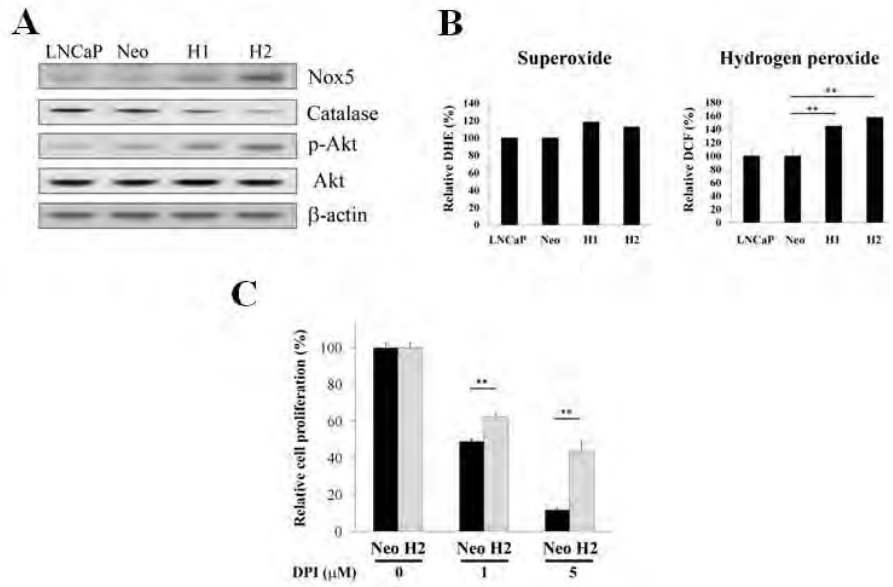

Fig. 5. SREBP-1 induces cell proliferation through alteration of ROS, Nox5 and catalase expression in prostate cancer cells. *A*, SREBP-1 induced Nox5 and inhibited catalase expression in prostate cancer cells (H1 and H2 clones) determined by Western blot. SREBP-1 also increased p-Akt expression, a survival signaling protein. β-actin was used as an internal loading control. *B,* SREBP-1 increased the levels of hydrogen peroxide (DCF, see Material and Methods) in H1 and H2 prostate cancer cells. The levels of superoxide (DHE) were not significantly changed by SREBP-1. The relative DCF (%, the right panel) and DHE (%, the left panel) were assigned as 100% in LNCaP cells. **, $P < 0.005$, significant differences from Neo cells. Data represent the mean ± SD of two independent two triplicate experiments. *C,* DPI, a Nox specific inhibitor and ROS scavenger, inhibited cell proliferation of Neo and H2 prostate cancer cells in a dose-dependent pattern (0 to 5 μM) during a two-day treatment. H2 cells with high Nox5 expression (Fig. 3A) and ROS status (Fig. 3B) increased the resistance of DPI-mediated suppression of cell proliferation. The relative cell proliferation (%) was assigned as 100% in each cell clones without DPI treatment. **, $P < 0.005$, significant differences from Neo cells at the same dose of DPI. Data represent the mean ± SD of two independent four replicate experiments.

levels of superoxide were not significantly changed by SREBP-1 (Fig. 5B, the left panel). Additionally, expression of catalase, a key enzyme of hydrogen peroxide degradation, decreased in SREBP-1 overexpresssing H1 and H2 cells (Fig. 5A). Down-regulation of catalase may also cause hydrogen peroxide accumulation in prostate cancer cells. To further investigate if SREBP-1 induces prostate cancer cell proliferation through activation of Nox5 and ROS, a Nox specific inhibitor, DPI, was used to treat with these prostate cancer cells. DPI has also been showed to decrease hydrogen peroxide levels in prostate cancer cells (16). As shown in Fig. 5C, cell proliferations of both Neo and H2 cells were affected by DPI in a concentration-dependent inhibition. However, overexpressing SREBP-1 H2 cells with high Nox5 expression (Fig. 5A) and ROS status (Fig. 5B) increased the resistance of DPI-mediated suppression of cell proliferation (Fig. 5C). These data indicated that SREBP-1 induced prostate cancer cell proliferation via Nox5 and ROS.

6) SREBP-1 promotes prostate tumor growth and castration resistance in a subcutaneous xenograft mouse models. Because SREBP-1 expression increased in advanced form of human prostate cancer (17), we

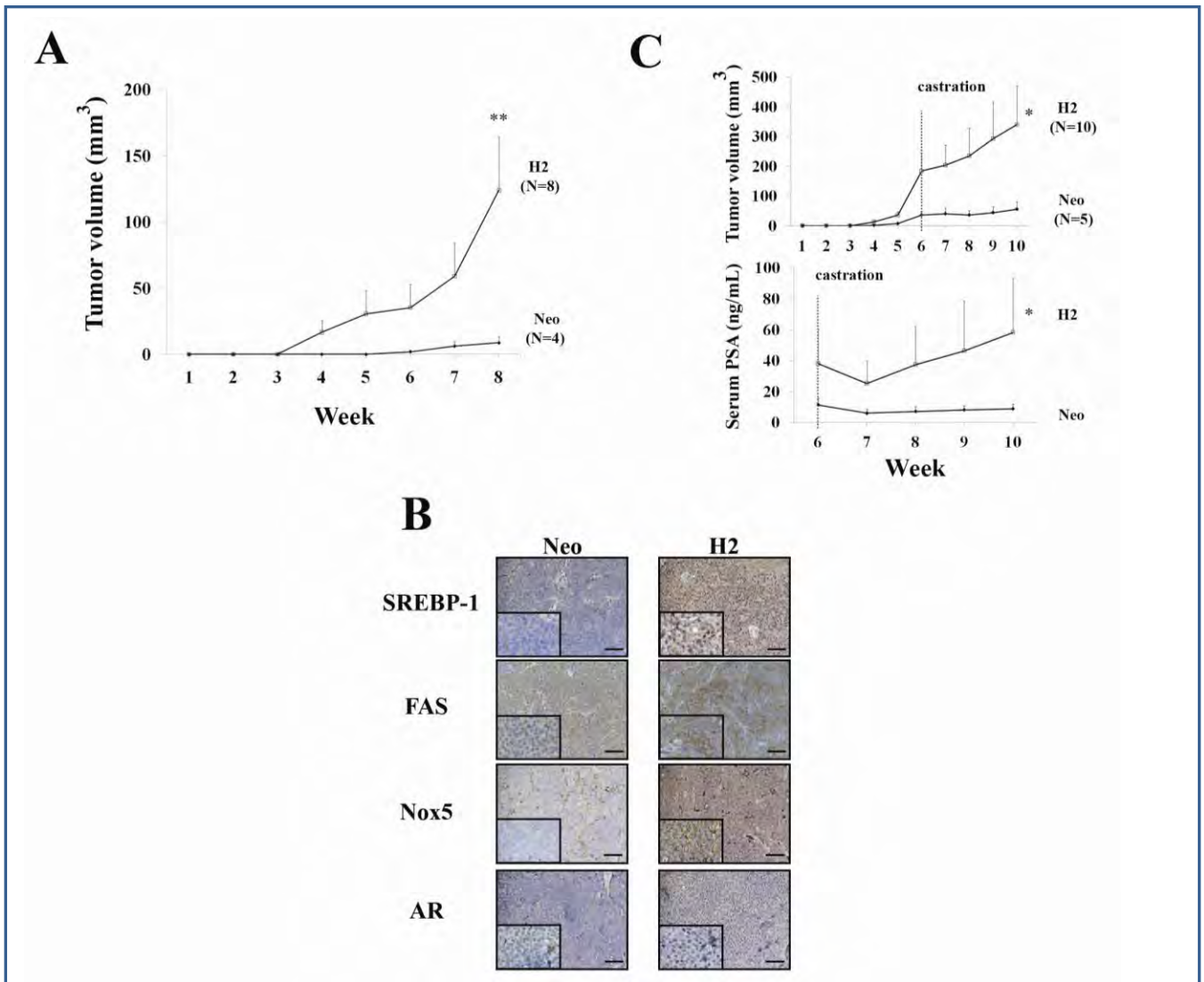

Fig. 6. SREBP-1 promotes human prostate tumor growth and castration resistance in mouse subcutaneous xenograft models. *A*, Tumor growth was assayed by tumor volume after inoculation of H2 and control Neo cells in mouse subcutaneous areas. SREBP-1 significantly induced the growth of H2 compared to Neo tumors. **, $P < 0.005$, significant differences from Neo tumors. *B*, IHC of subcutaneous Neo and H2 tumor specimens. H2 tumors highly expressed SREBP-1 (most in nuclei), FAS (cytoplasm), Nox5 (cell membranes) and AR (most in nuclei) proteins compared to Neo tumors. Scale bar = 100 μm. *C*, The mouse castration study. Tumor volumes of subcutaneous H2 tumors continuously increased after mouse castration (at week 6) compared to Neo tumors (the top panel). Serum PSA levels of both Neo and H2 tumor-bearing mice dropped at the first week post-castration (at week 7, the bottom panel). However, PSA levels of H2 mice significantly increased after four week castration (at week 10) compared to Neo group. *, $P < 0.05$, significant differences from Neo.

seek to determine if SREBP-1 confers growth advantages in hormone-naïve mice and resistance to tumor shrinkage in surgically castrated mice. We found SREBP-1 overexpressing H2 cells when inoculated subcutaneous developed 100% incidence of tumor formation (8/8) in mice; control Neo cells only exhibited 50% incidence of the tumor formation (4/8) during an 8-week of observation. LNCaP classically showed less aggressive and low tumorigenic characteristics in mouse models (18). Furthermore, H2 tumors exhibited a 14-fold increased growth rate over that of the Neo tumors, as assessed by tumor volumes (Neo: 8.8±5.0 mm^3 and H2: 124.0±40.0 mm^3), after 8-week *in vivo* growth (Fig. 6A). Consistent with previous Western blot results, IHC data showed that H2 highly expressed SREBP-1 (most in nuclei), FAS (cytoplasm), Nox5 (cell membranes) and AR (most in nuclei) in comparison to Neo tumors harvested from mouse subcutaneous space (Fig. 6B). Next, we sought to determine if SREBP-1 would be able to mediate castration resistance in prostate tumor xenografts grown in mice. Upon castration (at week 6), strikingly, subcutaneous H2 tumor growth continued compared to Neo tumors (Fig. 6C, the top panel). Serum PSA levels of both Neo and H2 tumor-bearing mice dropped at the first week post-castration (at week 7). However, serum PSA levels of H2 mice significantly rebounded after four weeks of castration (at week 10) compared to Neo mice (Fig. 6C, the bottom panel). These results suggested that SREBP-1 regulates prostate tumor occurrence, growth, and even resistance to castration in mice.

7) A new fat and weight reducing agent, 125B11, regulated gene expression and inhibited cell proliferation through blockade of SREBP-1 nuclear translocation in prostate cancer cells. A new small synthetic molecule, 125B11, has been reported to specifically inhibit SREBP-1 nuclear translocation and it downstream target gene expression, and further reduced fat and body weight in obese mice (19). First, we determined whether SREBP-1 nuclear translocation was affected by 125B11 in human prostate cancer cells. 125B11 inhibited nuclear translocation of mature SREBP-1 (68 kDa), and its target gene expression, including FAS, Nox5 and AR (20), in both Neo and H2 cells (Fig. 7A). Next, we sought to examine if blocking SREBP-1 nuclear translocation by 125B11 affected cell proliferation in prostate cancer cells. As shown in Fig. 7B, 125B11 decreased cell proliferation of Neo and H2 cells with a dose-dependent pattern. These data suggest that by inhibition of SREBP-1 nuclear translocation, 125B11 decreased expressions of Nox5, FAS and AR, and reduced cell proliferation in prostate cancer cells.

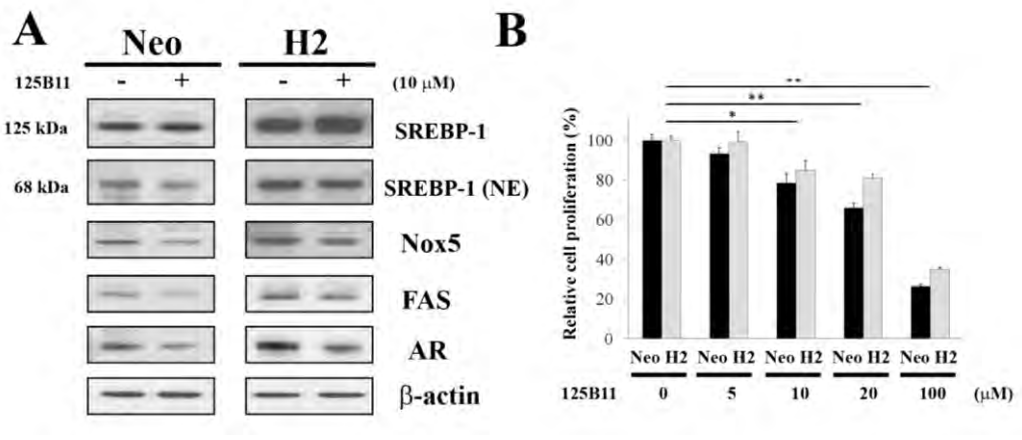

Fig. 7. A novel fat and weight reducing agent, 125B11, regulated gene expression and inhibited cell proliferation through blockade of SREBP-1 nuclear translocation in prostate cancer cells. *A*, 125B11 inhibited nuclear translocation of mature SREBP-1 (68 kDa), and expressions of Nox5, FAS and AR in both Neo and H2 cells. *B*, 125B11 affected cell proliferation (2-day) of Neo and H2 in a dose-dependent inhibition (0 to 100 μM). *, $P < 0.05$; and **, $P < 0.005$.

8) 125B11 induced apoptotic death in prostate cancer cells—To investigate if blockade of SREBP-1 activity by 125B11 will induce apoptotic death in prostate cancer cells, we examined caspase expression by Western blot in Neo and H2 LNCaP cells. The results of Western blot analysis of caspases showed that cleaved caspase-9, caspase-3, and PARP were increased by exposing Neo and H2 cells to 125B11 (Fig. 8). Collectively (Figs 7 and 8) indicate that through interrupting SREBP-1 activity by a new agent, 125B11, decreased expressions of

SREBP-1 downstream target genes, *Nox5*, *FAS* and *AR*, reduced cell proliferation and induce apoptotic death in prostate cancer cells.

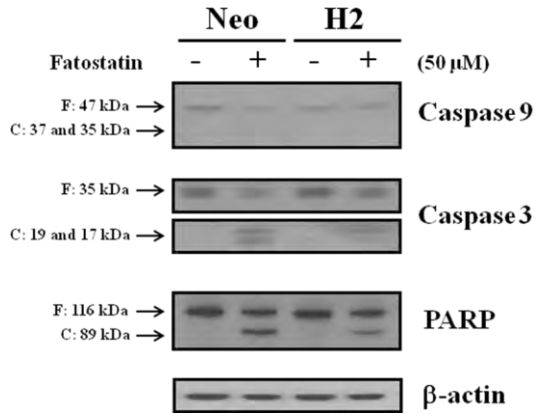

Fig. 8. 125B11 induced cell death of prostate cancer cells through an apoptotic cascade pathway. 125B11 (0 to 10 μg/mL, 48 h treatment) activated the expression of cleaved caspase-9, caspase-3 and PARP proteins in Neo and H2 LNCaP cells as assayed by Western blot. F: full-length form; C: cleavage forms

KEY RESEARCH ACCOMPLISHMENTS:

- Blockade of β2M using β2M mAb significantly downregulated AR and PSA expression and induced an apoptotic caspase-dependent pathway in prostate cancer cells.
- We identified a *cis*-acting element, SREBP-1 binding site, within the 5'-flanking promoter region of hAR gene is responsible for AR transcriptional activity regulated by β2M mAb.
- We further demonstrated that a transcription factor, SREBP-1, interacting with SREBP-1 binding site within the hAR promoter region mediated by β2M mAb in prostate cancer cells.
- SREBP-1 plays a key role in regulation of AR expression and cell viability in prostate cancer cells.
- SREBP-1 increase fatty acid, lipid and cholesterol accumulation and oxidative stress in prostate cancer cells
- SREBP-1 promotes tumor initiation, burden and castration resistance of human prostate tumors in mouse subcutaneous xenograft models.
- Target SREBP-1 by a novel fat and body weight lowing agent, 125B11, provides a new and promising therapeutic approach to prevent and treat prostate cancer malignancy.

REPORTABLE OUTCOMES:

First year of this DoD geant from May 1, 2008 to April 30, 2009: We published a peer-reviewed research article in *Clinical Cancer Research* (14: 5341-7, 2008, PMCID: PMC3032570) and presented a poster presentation in 2009 AACR annual meeting (# 849; title: Anti-β2-microglobulin monoclonal antibody inhibition of androgen receptor expression and survival through a lipogenic pathway in prostate cancer).

Second year of this DoD geant from May 1, 2009 to Dec 31, 2010: we published another peer-reviewed research article in *J Biol Chem* (285: 7947-59, 2010, PMCID: PMC2832945) and presented a poster presentation in 2010 AACR annual meeting.

Three year of this DoD geant from Jan 1, 2011 to January 2, 2012: I presented a poster presentation in 2011 IMPaCT meeting (# PC073356-1798; title: A novel anti-β2-microglobulin antibody inhibition of androgen receptor expression, survival and progression in prostate cancer cells). Currently, we are preparing the third paper regarding "the SREBP-1/AR/lipogenesis/oxidative stress study" for this award.

CONCLUSION:

β2M is a signaling and growth-promoting factor inducing prostate cancer cell proliferation, survival and progression. Interrupting β2M and its related signaling pathways by a novel agent, β2M mAb resulted in the inhibition of AR and PSA expression and the induction of apoptosis of prostate cancer cells. The molecular mechanism of AR inhibitory expression by β2M mAb was through decreasing the interaction between a

lipogenic transcription factor, SREBP-1, and its binding *cis*-acting element located in the 5'-flanking AR promoter region determined by electrophoretic mobility shift assay and chromatin immunoprecipitation assay. The functional study of SREBP-1 revealed that knocked-down or overexpressed SREBP-1 by utilizing a sequence-specific siRNA or an expression vector showed to decrease or increase total and nuclear AR protein in prostate cancer cells. SREBP-1 also induced *in vitro* cell proliferation, migration and invasion in prostate cancer cells. Additionally, SREBP-1 induced oxidative stress through increase of ROS levels and Nox5 expression in prostate cancer cells. ROS are reactive molecules produced in cells when oxygen is metabolized, including superoxide and hydrogen peroxide, which have been shown to induce and activate intracellular signaling pathways, cancer development and progression. In subcutaneous xenograft mouse models, strikingly, SREBP-1 increased LNCaP tumor intiation and burdens and promoted castration-resistant progression of prostate tumor. In summary, β2M mAb is a potent and attractive pleiotropic therapeutic agent to inhibit AR expression, cell proliferation, survival and fatty acid and lipid metabolism through down-regulation of a lipogenic transcription factor, SREBP-1, in prostate cancer cells. Targeting SREBP-1 also provides an alternative therapeutic approach for prostate cancer.

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β2-Microglobulin Signaling Blockade Inhibited Androgen Receptor Axis and Caused Apoptosis in Human Prostate Cancer Cells

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Abstract

Purpose: β2-Microglobulin (β2M) has been shown to promote osteomimicry and the proliferation of human prostate cancer cells. The objective of this study is to determine the mechanism by which targeting β2M using anti-β2M antibody inhibited growth and induced apoptosis in prostate cancer cells.

Experimental Design: Polyclonal and monoclonal β2M antibodies were used to interrupt β2M signaling in human prostate cancer cell lines and the growth of prostate tumors in mice. The effects of the β2M antibody on a survival factor, androgen receptor (AR), and its target gene, *prostate-specific antigen* (*PSA*) expression, were investigated in cultured cells and in tumor xenografts.

Results: The β2M antibody inhibited growth and promoted apoptosis in both AR-positive and PSA-positive, and AR-negative and PSA-negative, prostate cancer cells via the down-regulation of the AR in AR-positive prostate cancer cells and directly caused apoptosis in AR-negative prostate cancer cells *in vitro* and in tumor xenografts. The β2M antibody had no effect on AR expression or the growth of normal prostate cells.

Conclusions: β2M downstream signaling regulates AR and PSA expression directly in AR-positive prostate cancer cells. In both AR-positive and AR-negative prostate cancer cells, interrupting β2M signaling with the β2M antibody inhibited cancer cell growth and induced its apoptosis. The β2M antibody is a novel and promising therapeutic agent for the treatment of human prostate cancers.

β2-Microglobulin (β2M) is produced by all nucleated cells as a 119-amino-acid residue protein and, after processing, is secreted in a 99-amino-acid form (11,800 Da; refs. 1, 2). The most common known function of β2M, a light-chain antigen-presenting molecule, is to serve as a coreceptor for the presentation of the MHC class I in nucleated cells for cytotoxic T-cell recognition (3). However, cancer cells frequently down-regulate the expression of MHC class I to evade recognition by the immune system (4–7), presumably allowing the secretion of free β2M into circulation or in the tumor microenvironment. Our laboratory first identified β2M, an active component secreted by prostate cancer, and prostate and bone stromal cells, as a major growth factor and signaling molecule (8). β2M conferred osteomimicry, the ability of cancer cells to mimic gene expression by bone cells, in prostate cancer cells through the activation of a cyclic AMP (cAMP)–dependent protein kinase A (PKA) and cAMP-responsive element binding (CREB) protein signaling pathway (9). The use of a sequence-specific small interfering RNA (siRNA) targeting β2M and its signaling resulted in extensive prostate cancer cell death *in vitro* and greatly promoted prostate tumor regression in immunocompromised mice (8). We also showed that interrupting β2M signaling similarly blocked human renal cell carcinoma growth (10). β2M has recently been shown to be a useful biomarker for advanced human prostate cancer (11). β2M seems to be a downstream androgen target gene, more specific than prostate-specific antigen (PSA), under the control of the androgen receptor (AR), in a human LNCaP prostate cancer cell line (11).

Anti–β2M antibody is a potent interrupter of β2M-mediated signaling (8, 12). The β2M antibody was shown to be a highly cytotoxic reagent against the growth of solid tumors like renal cell carcinoma (13) as well as liquid tumors, such as leukemia, lymphoma, and multiple myeloma (12). We showed here that the β2M antibody inhibited the expression of a survival factor, AR, and its target gene, *PSA*, in AR-positive and PSA-positive human prostate cancer cell lines, including androgen-dependent LNCaP and androgen-independent C4-2B cells (14),

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and in androgen-independent C4-2 tumor xenograft models. The β2M antibody also suppressed growth and induced apoptosis in both AR-positive and PSA-positive, and AR-negative and PSA-negative human prostate cancer cells and in xenograft tumors in mice. Moreover, our studies showed that the β2M antibody induced prostate cancer cell death through an activation of a caspase-9–mediated apoptotic cascade pathway without affecting normal or nontumorigenic prostatic epithelial and stromal cells. These results support the idea that targeting β2M signaling via the external application of the β2M antibody can profoundly alter intracellular cell signaling networks, including, but not limited to, the AR downstream signaling axis. Effective β2M antibody–mediated targeting of the growth of both AR-positive and PSA-positive, and AR-negative and PSA-negative human prostate cancer cells may prove to be an attractive and safe therapeutic approach for the treatment of human prostate cancer and its lethal progression.

Materials and Methods

Cell lines, cell culture, and β2M antibody. The human prostate cancer cell line LNCaP (androgen dependent), the LNCaP lineage–derived bone metastatic subline C4-2B (androgen independent; ref. 14), DU-145 (brain metastatic, androgen independent), PC3 (bone metastatic, androgen independent), and ARCaP (ascites-fluid–derived, androgen repressive; refs. 15, 16) were cultured in T-medium (Invitrogen) supplemented with 5% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin. A human normal/nontumorigenic prostatic epithelial cell line, RWPE-1 (American Type Culture Collection), was cultured in keratinocyte serum-free medium supplemented with 5 ng/mL human recombinant epidermal growth factor and 0.05 mg/mL bovine pituitary extract (Invitrogen). These prostate cancer and normal cell lines were maintained in 5% CO_2 at 37°C. The anti-β2M antibody, a polyclonal antibody, was obtained from Santa Cruz Biotechnology, Inc. (sc-15366), for *in vitro* cell culture studies and *in vivo* animal experiments. We also tested the β2M monoclonal antibody (Santa Cruz Biotechnology; sc-13565) and found it to have similar inhibitory effects on the growth of human prostate cancer cells *in vitro* (data not shown).

Reverse transcription-PCR. LNCaP and C4-2B cells were plated on six-well dishes at 3×10^5 cells per well and grown to 70% confluence in T-medium with 5% fetal bovine serum. The cells were gently washed with PBS and incubated in T-medium plus 5% dextran-coated, charcoal-treated fetal bovine serum for overnight incubation. The cells were then treated with 0, 1, 5, or 10 μg/mL of β2M antibody; the β2M antibody was preincubated for 30 min with the same amounts of purified human β2M protein (Sigma) or 10 μg/mL of isotype control IgG for 24 h. The total RNA was isolated from these treated cells using a RNeasy Mini Kit (Qiagen) and subjected to reverse transcription according to the manufacturer's instructions (Invitrogen). The primer sequences used for PCR analysis were AR [5′-ATGGCTGTCATTCAGTACTCCTGGA-3′ (forward) and 5′-AGATGGGCTTGACTTTCCCAGAAAG-3′ (reverse)], PSA [5′-ATGTGGGTCCCGGTTGTCTTCCTCACCCTGTC-3′ (forward) and 5′-TCAGGGGTTGGCCACGATGGTGTCCTTGATC-3′ (reverse)], and glyceraldehyde-3-phosphate dehydrogenase [5′-ACCACAGTCCATGCCATCA-3′ (forward) and 5′-TCCACCACCCTGTTGCTGT-3′ (reverse)], respectively. The thermal profiles for AR, PSA, and glyceraldehyde-3-phosphate dehydrogenase cDNA amplification are 25 cycles, starting with denaturation for 1 min at 94°C, followed by 1 min of annealing at 61°C (for AR), 55°C (for PSA), and 60°C (for glyceraldehyde-3-phosphate dehydrogenase), and 1 min of extension at 72°C. The reverse transcription-PCR products were analyzed by agarose gel electrophoresis.

Western blot analysis and ELISA. Cell lysates were prepared from β2M monoclonal antibody–treated or IgG-treated prostate cells using a lysis buffer [50 mmol/L Tris (pH 8), 150 mmol/L NaCl, 0.02% NaN_3, 0.1% SDS, 1% NP40, and 0.5% sodium deoxycholate] containing 1 mmol/L phenylmethylsulfonyl fluoride and a protease inhibitor cocktail (Roche Applied Science). The protein concentration was determined by the Bradford assay using the Coomassie Plus Protein Reagent (Pierce). Western blot was done with the Novex system (Invitrogen) as described previously (8, 10). The primary antibodies anti-AR (1:500 dilution) and PSA (1:1,000 dilution; Santa Cruz Biotechnology); anti–caspase-9, caspase-3, and poly(ADP)ribose polymerase (PARP; 1:1,000 dilution; Cell Signaling Technology); and the secondary antibodies that were conjugated with horseradish peroxidase (1:5,000 dilution; GE Healthcare) were used. The detection of protein bands was done with the use of enhanced chemiluminescence Western Blotting Detection Reagents (GE Healthcare). The soluble PSA levels were determined by microparticle ELISA with the Abbott IMx machine (Abbott Laboratories).

Cell proliferation assay. LNCaP (6,000 cells per well), C4-2B (6,000 cells per well), DU-145 (3,000 cells per well), PC3 (3,000 cells per well), ARCaP (5,000 cells per well), and RWPE-1 (6,000 cells per well) cells were plated on 96-well plates and treated with the β2M antibody or control IgG for a 3-d incubation. The cell numbers were measured every 24 h by mitochondrial 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS), assay with the use of the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions.

Sub-G_1 DNA content measurement. LNCaP and C4-2B prostate cancer cells were plated on six-well plates at 3×10^5 per well in T-medium containing 5% dextran-coated, charcoal-treated fetal bovine serum and exposed to 10 μg/mL of β2M monoclonal antibody or control IgG for 48-h incubation. The treated cells were collected by trypsinization and fixed in 70% ice-cold ethanol, incubated with RNase A (100 μg/mL; Sigma) for 30 min, and stained with propidium iodide (25 μg/mL; Chemicon) for 30 min. The cell cycle was determined by a FACScan flow cytometer and CellQuest software (Becton Dickinson Labware) for analysis of sub-G_1 DNA content.

In vivo *animal experiments.* All the animal experiments were approved and done in accordance with institutional guidelines. The mice were maintained at the Animal Research Facility in Emory University. To test the antitumor efficacy and AR expression regulated by the β2M antibody *in vivo*, 4-wk-old male athymic *nu/nu* mice (National Cancer Institute) were inoculated s.c. with C4-2 or PC3 prostate cancer cells with 2×10^6 cells per mouse. After 3 wk (PC3 tumor) or 4 wk (C4-2 tumor) of inoculation, 10 μg of β2M monoclonal antibody mixed with Surgifoam (Ethicon Inc.) to keep and slow release the β2M antibody around the tumors were given by intratumoral implantation, one shot per mouse. The control group mice received equal doses of isotype IgG or placebo (saline) implanted the same way as the β2M antibody. After 1 wk of treatment, tumor tissues were harvested from the euthanized mice and fixed in 10% formalin, dehydrated in ethanol, embedded in paraffin, and sectioned in slides. The blank tissue slides were subjected to immunohistochemical staining with anti-AR antibody (Santa Cruz Biotechnology) and M30 CytoDeath marker (DiaPharma Group, Inc.), and detected by the Dako Autostainer Plus system (Dako Corp.). For quantification of AR and M30 CytoDeath staining, 100 cells at five randomly selected areas were counted and the positive-staining cells were recorded.

Statistical analysis. Statistical analyses were done as described previously (9). Student's *t* test and two-tailed distribution were applied in the analysis of statistical significance.

Results

β2M antibody decreased AR and PSA expression in human prostate cancer cells. We previously showed that β2M is a novel signaling and growth-regulating molecule capable of

promoting cell proliferation and survival in human prostate and renal cancer cells (8, 10). Interrupting β2M and its downstream signaling by β2M siRNA induced cell death in both human prostate and renal carcinoma models (8, 13). Because the downstream targets for β2M signaling interruption are not completely clear in human prostate cancer cells, we conducted a cDNA microarray study (17) comparing β2M siRNA stably transfected AR-positive and PSA-positive C4-2B prostate cancer cells with their scramble stably transfected control clones. The results of these studies showed a 4-fold and 16-fold decreased expression of AR and PSA mRNA, respectively, in C4-2B cells, and these data were confirmed by reverse transcription-PCR and Western blot.[5] To test the hypothesis that blocking β2M-mediated signaling pathways may affect AR gene expression and transactivation, which are involved in prostate cancer cell growth, survival, and progression, we tested the effect of a new reagent, β2M polyclonal antibody, on AR and PSA expression in AR-positive and PSA-positive LNCaP (androgen dependent) and C4-2B (androgen independent) cells. Consistent with cDNA microarray data, interrupting β2M by the β2M antibody decreased endogenous AR and PSA mRNA expression as determined by reverse transcription-PCR (Fig. 1A). The inhibitory effect of the β2M antibody (0-10 μg/mL) was concentration dependent, and the addition of purified β2M protein rescued the decreased AR and PSA mRNA expression that had been inhibited by the β2M antibody in LNCaP and C4-2B cells. Isotype-matched control IgG (10 μg/mL) did not suppress AR and PSA mRNA expression. In parallel, the β2M antibody (0-10 μg/mL) also inhibited AR and PSA protein levels in a concentration-dependent manner as analyzed by Western blot (Fig. 1B), and this inhibition can also be rescued by the addition of purified β2M protein to the cultured LNCaP and C4-2B cells. The control IgG did not change AR and PSA protein expression. Consistent with the blockade of AR expression, we found that secreted soluble PSA levels, assayed by ELISA, were also decreased by the β2M antibody, but not the control IgG, in LNCaP and C4-2B cells (Fig. 1C). These results indicate that the β2M antibody diminished AR and PSA mRNA and protein expression in both androgen-dependent and androgen-independent human prostate cancer cells.

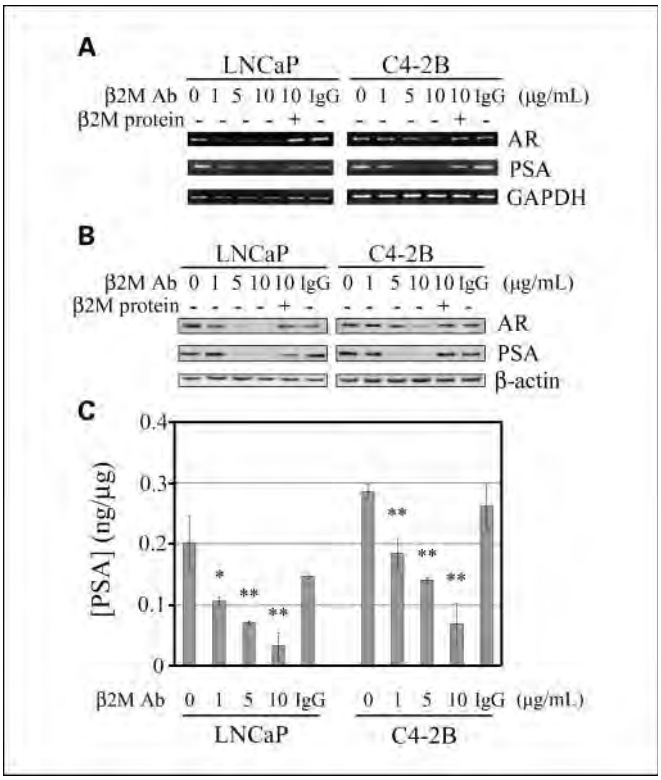

Fig. 1. β2M antibody inhibited AR and PSA mRNA and protein expression in human prostate cancer cells. *A,* β2M antibody (*β2M Ab*) decreased AR and PSA mRNA expression in a dose-dependent manner (0-10 μg/mL, 24-h treatment) in both LNCaP (androgen dependent) and C4-2B (androgen independent) prostate cancer cell lines detected by reverse transcription-PCR. The inhibitory effect was restored by the preincubation of the β2M antibody with purified β2M protein. Isotype control IgG (10 μg/mL) did not affect AR and PSA mRNA expression. The expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a loading control. *B,* β2M antibody inhibited AR and PSA protein expression in a dose-dependent pattern (0-10 μg/mL, 24-h treatment) in LNCaP and C4-2B cells assayed by Western blot. The inhibitory effect was abrogated by the preincubation of β2M antibody with β2M protein. Control IgG (10 μg/mL) did not change AR and PSA protein expression. β-Actin was used as an internal loading control. *C,* secreted soluble PSA levels were also decreased by the β2M antibody (0-10 μg/mL), but not the control IgG, in a concentration-dependent inhibition in LNCaP and C4-2B cells determined by ELISA. The concentrations of PSA (ng) were normalized by total proteins (μg). *, $P < 0.05$; **; $P < 0.005$, significant differences from the β2M-antibody – untreated group. Columns, mean; bars, SD.

β2M antibody inhibited cell proliferation in human prostate cancer cell lines. Because β2M stimulated prostate and renal cancer cell growth through the promotion of cAMP/PKA/CREB signaling pathway and the activation of cyclins and cell cycle progression (8, 10), we investigated the possibility that interrupting the β2M-mediated signaling axis may be cytotoxic to prostate cancer cells. When the LNCaP and C4-2B cells were exposed to the β2M antibody (0-20 μg/mL) for a 2-day incubation, the growth of these two prostate cancer cell lines was inhibited in a concentration-dependent manner, with an IC_{50} of 10.3 and 7.4 μg/mL, respectively (Fig. 2A). The purified β2M protein was shown to rescue the β2M antibody–induced inhibition of prostate cancer cell proliferation, whereas the control IgG did not affect the growth of the LNCaP and C4-2B cells (Fig. 2A). Because of the AR heterogeneity in human prostate cancer cells (18), we compared the effects of the β2M antibody on the cell proliferation of AR-positive (LNCaP, C4-2B, and ARCaP) and AR-negative (PC3 and DU-145) human prostate cancer cell lines. Figure 2B shows that the β2M antibody (10 μg/mL) inhibited the proliferation of these prostate cancer cells at day 3 by 57% (LNCaP), 82% (C4-2B), 91% (DU-145), 93% (PC3), and 94% (ARCaP). These data suggest that the β2M antibody significantly inhibited cell proliferation in a broad range of human prostate cancer cell lines.

β2M antibody induced apoptotic death and inhibited AR expression of prostate cancer cells* in vitro *and in mouse xenograft models. To determine the molecular mechanism by which the β2M antibody inhibited the growth of prostate cancer cells, we first examined apoptotic death in LNCaP and C4-2B cells, including sub-G_1 DNA content analysis and activation of caspase (19) and PARP expression. The results of flow cytometric analysis revealed that the β2M antibody greatly increased sub-G_1 DNA contents in LNCaP (% sub-G_1 = 82.49) and C4-2B (% sub-G_1 = 79.45) cells compared with the control IgG-treated LNCaP (% sub-G_1 = 0.86) and C4-2B (% sub-G_1 = 0.54) cells (Fig. 3A). Western blot analysis of

[5] Unpublished data.

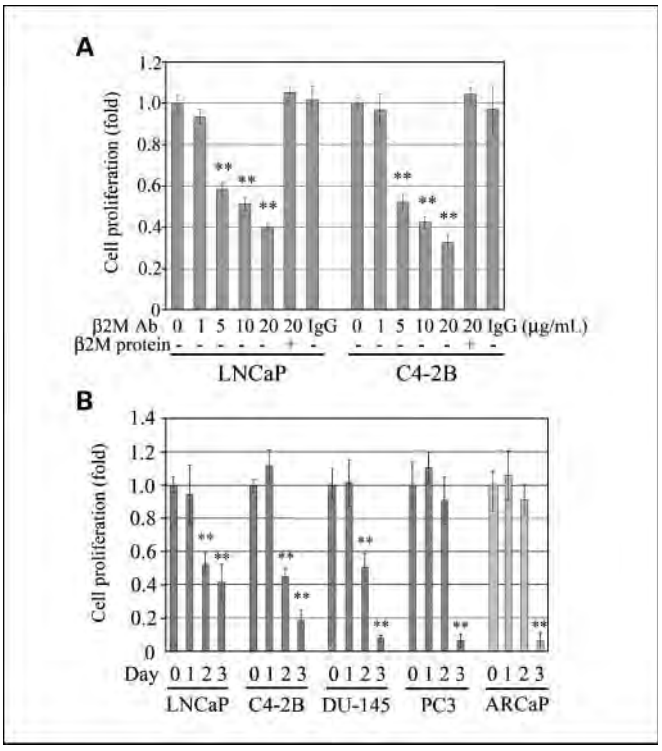

Fig. 2. β2M antibody inhibited the growth of prostate cancer cell lines. *A,* β2M antibody significantly affected the cell proliferation of LNCaP and C4-2B prostate cancer cells, with a dose-dependent inhibition (0-20 μg/mL) after 2-d incubation determined by mitochondrial MTS assay (Promega). Purified β2M protein rescued the inhibitory effect on cell growth regulated by the β2M antibody. IgG (20 μg/mL) did not decrease the growth of LNCaP and C4-2B cells. The relative fold was assigned as 1.0 in the absence of β2M antibody treatment. **, $P < 0.005$, significant differences from the β2M-antibody – untreated group. Columns, mean of five replicate experiments; bars, SD. *B,* β2M antibody (10 μg/mL) inhibited the cell proliferation of a broad range of human prostate cancer cell lines, LNCaP, C4-2B, DU-145, PC3, and ARCaP, during 3-d treatment. The cell numbers were measured daily with a mitochondrial MTS method. The relative fold was assigned as 1.0 at day 0 for each prostate cancer cell line. **, $P < 0.005$, significant differences from day 0 for each cell line. Columns, mean of four or five replicate experiments; bars, SD.

caspases showed that cleaved caspase-9, caspase-3, and PARP, a downstream factor of caspases, were increased by exposing the LNCaP and C4-2B cells to the β2M antibody, but not the control IgG, for a 48-h incubation (Fig. 3B). The induction of cleaved caspases and PARP was attenuated by the preincubation of the β2M antibody with purified β2M protein. In addition, cell death induced by the β2M antibody was also confirmed at the level of light microscopy in LNCaP and C4-2B cells (Fig. 3C).

Next, we examined the effects of the β2M antibody on cell death and/or the status of AR in preexisting C4-2 (AR positive) and PC3 (AR negative) prostate tumors grown in mice as subcutaneous xenografts, with the antibody delivered as Surgifoam implants, and isotype-matched IgG and saline delivered similarly as controls. After 1-week treatment, tumor tissues were harvested from the euthanized mice and subjected to immunohistochemical staining of the AR and a commercially available cell death marker, M30 CytoDeath. Figure 4A and B shows that the β2M antibody dramatically inhibited AR expression in C4-2 tumors and induced cell death in both C4-2 and PC3 tumors in mice compared with the IgG-treated and saline-treated controls. The cell numbers of positive AR staining in the β2M-antibody–treated C4-2 tumor xenografts were greatly decreased from 81 ± 6 per 100 cells (IgG controls) and 76 ± 4 per 100 cells (saline controls) to 10 ± 3 per 100 cells. Markedly increased prostate cancer death from the β2M antibody was observed in both C4-2 (the positive M30 CytoDeath staining cells were 36 ± 8 cells per 100 cells) and PC3 (55 ± 15 cells per 100 cells) tumor specimens compared with the IgG-treated (C4-2, 9 ± 2 cells per 100 cells; PC3, 16 ± 3 cells per 100 cells) and saline-treated (C4-2, 10 ± 3 cells per 100 cells; PC3, 13 ± 2 cells per 100 cells) control groups.

We further investigated whether the β2M antibody may be a safe reagent to selectively kill cancer but not normal or nontumorigenic immortalized cell lines. A human nontumorigenic prostatic epithelial cell line, RWPE-1, was exposed to the β2M antibody and the control IgG. In contrast to human prostate cancer cells, the β2M antibody did not inhibit RWPE-1 cell growth (Fig. 5A), did not decrease its endogenous AR expression (Fig. 5B), and did not activate apoptotic marker expression as assayed by Western blot (Fig. 5B). While the β2M antibody showed low cytotoxicity in RWPE-1 cells, it also did not affect the growth of P69, a SV40-immortalized human normal prostatic epithelial cell line (20), and human normal prostatic stromal cells (data not shown).

In summary, our results collectively indicate that the β2M antibody effectively induced human prostate cancer, but not normal prostate, cell apoptosis in culture. The β2M antibody induced cell death in prostate tumor xenografts in mice regardless of their AR status. The β2M antibody was also shown to down-regulate AR and PSA expression in AR-positive and PSA-positive human prostate cancer cells grown in culture and as subcutaneous xenografts in mice.

Discussion

Prostate cancer progression from an androgen-dependent to an androgen-independent state symbolizes its hormone-refractory status and occurs in patients clinically. Because there is currently no effective therapy for the management of hormone-refractory prostate cancer, we undertook the investigation of the molecular mechanisms and effects of a recently identified novel molecular target, β2M, using β2M antibody as a single agent in experimental models of human prostate cancer. Our results showed that the β2M antibody exerted growth inhibitory and apoptotic action in AR-positive and PSA-positive human prostate cancer cells. The β2M antibody was also shown to induce similar apoptotic death in AR-negative and PSA-negative, and androgen-unresponsive human prostate cancer cells. Because aberrant androgen signaling mediated by the AR, a ligand-activated transcription factor and a survival factor, plays a key role in regulating prostate cancer growth and survival even in cells that are considered as androgen refractory (21, 22), we investigated the effects of the β2M antibody on the AR-signaling axis based on a cDNA microarray study, in which targeting β2M was shown to markedly down-regulate AR and PSA in AR-positive human prostate cancer cells C4-2B. Our results confirmed that the β2M antibody blocked AR signaling and PSA production in a series of AR-positive and PSA-positive, and lineage-related LNCaP (androgen dependent), C4-2 (androgen independent), and C4-2B (androgen independent) cells in a β2M-dependent manner (i.e., β2M protein could rescue the inhibitory effects of the β2M antibody).

We previously reported that a small protein, β2M, which was considered as a "housekeeping" gene product (23), was a key growth and signaling molecule regulating osteomimicry and promoting growth and survival in prostate cancer cells (8, 9). Targeting β2M and its signaling by β2M siRNA greatly induced prostate cancer cell death both in cultured cells and in mice with preestablished human prostate tumors (8). In the present study, we used the β2M antibody to block β2M-related signaling pathways, hoping to induce apoptosis in prostate tumors and rationalize the exploration of the β2M antibody as a novel agent for clinical trial in men with hormone-refractory cancer. We showed that the β2M antibody as a single agent significantly inhibited AR and PSA mRNA and protein expression in both LNCaP and C4-2B cells and induced apoptotic cell death in prostate tumor cells *in vitro* and in mouse xenografts (C4-2 and PC3 tumors) *in vivo* regardless of their AR status. The selective ability of the β2M antibody to block prostate tumor growth without affecting normal or nontumorigenic cells, including human normal prostatic epithelial and stromal cells, and normal hematopoietic cells *in vitro* or other normal tissues *in vivo* (12), suggests that the β2M antibody is a cancer-specific targeting agent that can be applied in the treatment of human prostate cancers. This conclusion is supported by previous studies in which immune intact mice with β2M knockdown survived and developed mild degrees of iron overload and arthritis without compromising their life expectancy (24–26). In addition, during a 10-week observation period, we have not noted any toxicity in mice treated intratumorally with the β2M antibody as evaluated by their body weights and physical appearance (data not shown). This observation is concurred by the early report of Yang et al. (12) although additional work is warranted to test the potential cytotoxicity of this antibody in immune intact hosts. We envision, nevertheless, that the β2M antibody can be applied in a cyclic manner to patients with prostate cancer, allowing

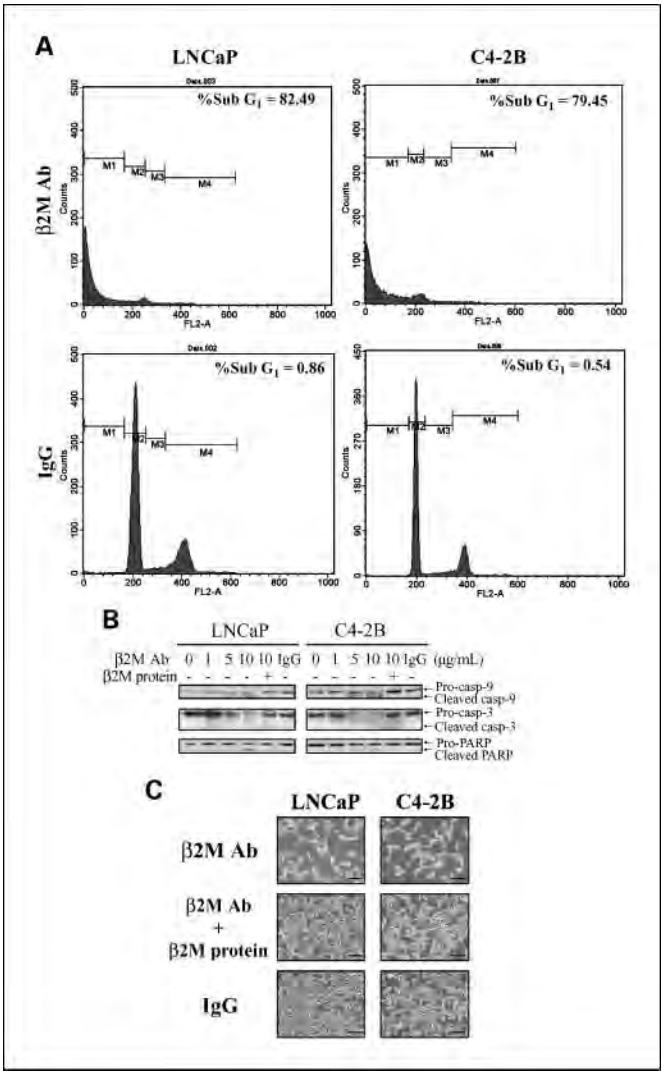

Fig. 3. β2M antibody induced the cell death of prostate cancer cells through an apoptotic cascade pathway. *A,* LNCaP and C4-2B cells were exposed to either the β2M antibody or isotype control IgG (10 μg/mL) for 48-h incubation and subjected to cell cycle analysis determined by flow cytometry. Both LNCaP and C4-2B cells treated with the β2M antibody showed a marked increase in the sub-G_1 DNA contents compared with IgG-treated cells. *B,* β2M antibody (0-10 μg/mL, 48-h treatment) activated the expression of cleaved caspase-9, caspase-3, and PARP proteins in a dose-dependent pattern in LNCaP and C4-2B cells as assayed by Western blot. β2M protein rescued the apoptotic effect of the β2M antibody. Control IgG (10 μg/mL) did not activate cleaved caspase and PARP expression. *C,* LNCaP and C4-2B cells were treated with the β2M antibody; the β2M antibody was preincubated with β2M protein or control IgG (10 μg/mL) for 48 h and examined by light microscopy. Bar, 250 μm.

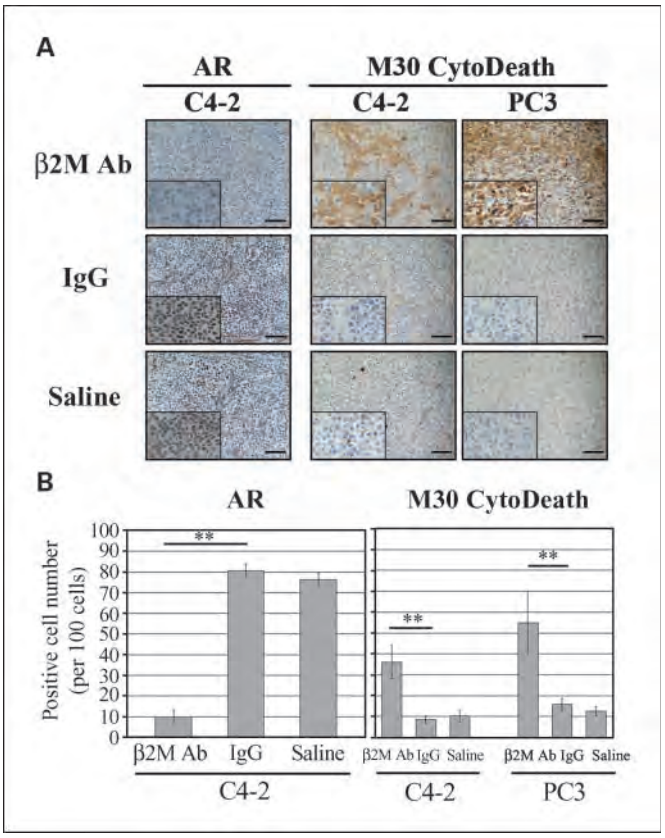

Fig. 4. β2M antibody decreased AR expression and induced the cell death of subcutaneous C4-2 and PC3 prostate tumor growth in athymic *nu/nu* mice. *A,* immunohistochemical analysis showed dramatic down-regulation of AR expression in β2M antibody – treated subcutaneous C4-2 tumor mouse xenografts ($n = 4$) but not in the control IgG-treated ($n = 4$) or saline-treated ($n = 4$) C4-2 tumor-bearing mice. The β2M antibody also markedly induced apoptotic death in both subcutaneous C4-2 ($n = 4$) and PC3 ($n = 4$) prostate tumors in xenograft mice assayed by M30 CytoDeath marker staining. Bar, 100 μm. *B,* quantification of the positive AR and M30 CytoDeath marker staining cells in C4-2 and PC3 tumor specimens from the immunohistochemical analysis (*A*). One hundred cells at five randomly selected areas were counted. **, $P < 0.005$, significant differences from the control IgG group. Columns, mean; bars, SD.

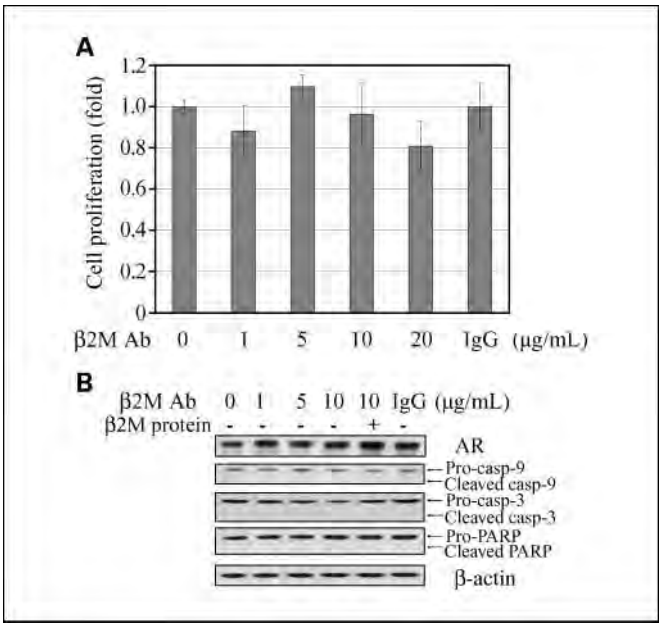

Fig. 5. β2M antibody did not affect cell proliferation and endogenous AR expression; it also did not induce apoptotic death in human normal/nontumorigenic prostatic epithelial cells. *A,* β2M antibody (0-20 μg/mL, 3-d incubation) did not significantly affect cell proliferation of human normal prostatic epithelial cells, RWPE-1, as determined by mitochondrial MTS assay. Control IgG (20 μg/mL) also did not affect the growth of RWPE-1 cells. The relative fold was assigned as 1.0 in the absence of β2M antibody treatment. Columns, mean of five replicate experiments; bars, SD. *B,* β2M antibody (0-10 μg/mL, 24-h treatment) did not inhibit AR nor activate cleaved caspase-9, caspase-3, and PARP protein expression in RWPE-1 cells assayed by Western blot. Control IgG (10 μg/mL) also did not affect AR, cleaved caspase, or PARP protein expression.

the immune system to return to normal function during the off-cycle of the β2M antibody application.

Other than the blockade of the β2M antibody on AR survival factor expression, the detailed molecular mechanisms by which the β2M antibody induced prostate cancer apoptosis are unclear. We previously showed that β2M promoted the expression of cell cycle markers, cyclin D1 and cyclin A, and cell growth in prostate cancer cells through the activation of a cAMP/PKA/CREB signaling pathway (8). We also showed that β2M stimulated renal cancer cell proliferation via the induction of phosphatidylinositol 3-kinase (PI3K)/Akt, mitogen-activated protein kinase (MAPK), and cAMP/PKA/CREB pathways (10). This pleiotropic cell signaling network activated by β2M is likely to be the target for the β2M antibody. It has been amply documented that the activation of AR, PI3K/Akt, and MAPK pathways are important features contributing to uncontrolled prostate cancer cell growth and survival (22, 27, 28). Indeed, we observed that the β2M antibody blocked not only the AR (Fig. 1A and B) but also the cell signaling network mediated by PI3K/Akt and MAPK pathways in LNCaP and C4-2B cells (Supplementary Fig. S1). These results are consistent with previous presentations that blocking β2M-mediated signaling pathways can interrupt the PI3K/Akt and MAPK signaling pathways and induce c-Jun-NH_2-kinase phosphorylation, resulting in the activation of a caspase-9–dependent apoptotic cascade in human renal cell carcinoma (13) and hematologic cancer cells (12). The constitutive activation of a PI3K/Akt signaling pathway has been shown in prostate cancer cell lines by the inactivation of the PTEN tumor suppressor (29). Because the PI3K/Akt signaling pathway has been reported to mediate AR mRNA and protein expression through AR promoter regulation (30), we anticipated that the β2M antibody inhibition of the PI3K/Akt and MAPK signaling pathways would cause growth retardation, apoptosis, and down-regulation of AR expression and activity in AR-positive and PSA-positive LNCaP/C4-2/C4-2B cells. Likewise, because of the blockade of these critical signaling pathways, we also expected diminished growth and induced apoptosis in AR-negative prostate cancer cells *in vitro* and *in vivo*. These results could have significant clinical implications. For example, the β2M antibody could be superior to other antiandrogenic therapies with actions that rely on intrinsic AR expression by prostate cancer cells. The β2M antibody could be used either as a single reagent or in combination with other therapeutic modalities for the treatment of both hormone-dependent and hormone-refractory prostate cancers because these have been shown to exhibit marked heterogeneity of AR expression (31). This approach is promising, considering recent success in the development of therapeutic antibodies (32), such as trastuzumab, a HER2/erbB2 antibody for breast cancers; bevacizumab, a vascular endothelial growth factor antibody; and cetuximab, an epidermal growth factor receptor antibody for metastatic colon cancers.

In summary, our investigation revealed for the first time that (*a*) the β2M antibody inhibited the expression of the AR and PSA in both androgen-dependent and androgen-independent AR-positive and PSA-positive human prostate cancer cells; (*b*) the β2M antibody has a broad spectrum of growth-inhibitory effects in both AR-positive and AR-negative prostate cancer cells; and (*c*) although the β2M antibody has been shown to be a potent pleiotropic signaling and growth inhibitor and to induce programmed cell death through a caspase-9–dependent pathway in prostate cancer cells, this antibody exhibited low cytotoxicity in human normal prostatic epithelial and stromal cells, which make it an attractive and safe therapeutic agent for future clinical application to treat prostate cancer and its progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Androgen Receptor Survival Signaling Is Blocked by Anti-β2-microglobulin Monoclonal Antibody via a MAPK/Lipogenic Pathway in Human Prostate Cancer Cells*[S]

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A new *cis*-acting element, sterol regulatory element-binding protein-1 (SREBP-1) binding site, within the 5′-flanking human androgen receptor (AR) promoter region and its binding transcription factor, SREBP-1, was identified to regulate AR transcription in AR-positive human prostate cancer cells. We further characterized the molecular mechanism by which a novel anti-β2-microglobulin monoclonal antibody (β2M mAb), shown to induce massive cell death in a number of human and mouse cancer cell lines, interrupted multiple cell signaling pathways in human prostate cancer cells. β2M mAb decreased AR expression through inactivation of MAPK and SREBP-1. By inactivation of MAPK, β2M mAb decreased prostate cancer cell proliferation and survival. By inhibition of SREBP-1, β2M mAb reduced fatty acid and lipid levels, an integral component of cell membrane, cell signaling mediators, and energy metabolism. These results provide for the first time a molecular link between the β2M intracellular signaling axis mediated by MAPK and SREBP-1 and involving lipid signaling, which collectively regulates AR expression and function. Antagonizing β2M by β2M mAb may be an effective therapeutic approach simultaneously targeting multiple downstream signaling pathways converging with MAPK, SREBP-1, and AR, important for controlling prostate cancer cell growth, survival, and progression.

β2-Microglobulin (β2M)[3] is a co-receptor of a major histocompatibility complex class I antigen. β2M has been implicated in the regulation of the host immune mechanism and is essential for the recognition of foreign antigens by T-lymphocytes (1). Recent reports from our laboratory and others assigned additional biological functions to β2M as a diagnostic and prognostic indicator for multiple myeloma, prostate, and breast cancers (2–5); a growth factor and a signaling molecule (6, 7); a new androgen and androgen receptor (AR) target gene (8); and an attractive new therapeutic target for both liquid (9) and solid (10, 11) tumor malignancies. Blockade of β2M and its related signaling pathways by sequence-specific siRNA or antibody resulted in the inhibition of AR expression and activity and the induction of extensive prostate cancer cell death *in vitro* as well as prostate tumor regression in immune-compromised mice (7, 10). In addition, anti-β2M monoclonal antibody (β2M mAb) has been shown not to significantly affect the growth of normal cells, consistent with experimental observations where transgenic mice with a β2M deficit had normal organ function and life expectancy (9, 10, 12). Therefore, β2M and its signaling axis may offer an opportunity for improving the clinical targeting of prostate cancer.

AR is a key growth and survival regulatory transcription factor for androgen target organs during normal development and neoplastic progression. Recognition of the importance of the AR signaling axis, particularly in castration-resistant prostate cancer, has prompted discoveries targeting androgen biosynthetic pathways using abiraterone as an agent for a Phase III trial (13, 14). Novel strategies to target AR directly through AR gene transcription and translation (10) or interfering in the interaction between AR and its co-factors and their downstream functions in prostate cancer cells have also been successfully attempted (15–17). AR activity is regulated by a host of factors including steroid hormones, thyroid hormones, vitamin D_3 (18), insulin-like growth factor I, insulin-like growth factor I receptor, keratinocyte growth factor, epidermal growth factor (19), interleukin-6 (20), and agents elevating and activating intracellular cAMP, G protein-coupled receptors, or a PKA signaling pathway (21, 22). The details of the transcriptional/translational mechanisms regulating AR within cancer cells remain unclear. Previous studies demonstrated that the 5′-flanking region of human AR promoter activity can be reg-

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[3] The abbreviations used are: β2M, β2-microglobulin; mAb, monoclonal antibody; AR, androgen receptor; ChIP, chromatin immunoprecipitation assay; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; FAS, fatty acid synthase; hAR, human androgen receptor; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; PSA, prostate-specific antigen; siRNA, small interfering RNA, SREBP-1, sterol regulatory element-binding protein-1; PI3K, phosphatidylinositol 3-kinase; reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EGR, early growth response gene; siβ2M, β2M siRNA; AP-1, activator protein-1.

ulated by transcription factors Sp1 (23), cAMP-responsive element-binding protein (24), FOXO3a (25), and lymphoid enhancer-binding factor-1/T cell-specific factor (LEF-1/TCF) (26), whose activities are subjected to modulation by several known cell signaling pathways such as cAMP/PKA, PI3K/Akt, MAPK, and Wnt/β-catenin in prostate cancer cells. In this study, we identified an additional transcription factor, SREBP-1, which affected lipid metabolism and accumulation, as a new downstream transcription factor under regulation by β2M mAb in prostate cancer cells.

SREBP-1 belongs to the SREBP family, which is a basic helix-loop-helix leucine zipper transcription factor (27, 28). Three major isoforms of SREBP have been identified, SREBP-1a, SREBP-1c, and SREBP-2 (28). SREBP-1 has been determined to regulate genes involved in fatty acid and cholesterol biosynthesis (27, 29), whereas SREBP-2 is more specific in the control of cholesterol metabolism (30). Dysregulation of SREBPs and their downstream regulated genes such as fatty acid synthase (FAS), which has been proposed to be a metabolic oncogene (31, 32), was shown to be involved in the development and progression of prostate cancer (33, 34). The expression of SREBP-1 was observed to be highly elevated in clinical human prostate cancer specimens compared with nontumor prostate tissues, and this may be relevant to androgen-refractory progression (34).

The objective of this study is to determine the pleiotropic β2M-mediated signaling mechanism by which a novel monoclonal antibody, β2M mAb, inhibited AR mRNA and protein expression and its transcription activity in AR-positive human prostate cancer cell lines. The results of this study suggest that β2M regulated multiple growth and survival signaling pathways through the control of transcription factors and their modifiers such as AR, MAPK, and PI3K/Akt (7, 10, 35). In particular, we demonstrated that marked down-regulation of AR as the consequence of targeting β2M by β2M mAb was due to the inactivation of a lipogenic transcription factor, SREBP-1, known to be associated with androgen-refectory progression of clinical prostate cancer (34). Accompanying reduction of SREBP-1 expression in prostate cancer cells, β2M mAb also decreased FAS expression and fatty acid and lipid levels, which are the main components of cell membrane and energy storage. Our data reveal for the first time a lipogenic pathway through MAPK and SREBP-1 that is critical for controlling AR expression, activity, and function in prostate cancer cells.

EXPERIMENTAL PROCEDURES

Prostate Cancer Cell Lines, Cell Culture, and Reagents—The LNCaP (androgen-dependent) human prostate cancer cell line and the LNCaP lineage-derived C4-2B bone metastatic subline (androgen-independent) were cultured in T-medium (Invitrogen) supplemented with 5% fetal bovine serum, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin. These prostate cancer lines were maintained in 5% CO_2 at 37 °C. β2M mAb was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Human SREBP-1 expression vector and SREBP-1 siRNA were obtained from OriGene Technologies, Inc. (Rockville, MD) and Santa Cruz Biotechnology, respectively. The selective inhibitors for signaling pathways of MAPK/ERK, U0126; PI3K, LY294002; and PKA, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89) were purchased from Cell Signaling Technology, Inc. (Beverly, MA).

RT-PCR—Total RNA was isolated from prostate cancer cells using a RNeasy kit (Qiagen). Total RNA was used as the template for RT according to the manufacturer's instructions (Invitrogen). The oligonucleotide primer sets used for PCR analysis of cDNA were as follows: β2M, 5′-ACGCGTCCGAAGCTTACAGCATTC-3′ (forward) and 5′-CCAAATGCGGCATCTAGAAACCTCCATG-3′ (reverse); AR, 5′-ATGGCTGTCATTCAGTACTCCTGGA-3′ (forward) and 5′-AGATGGGCTTGACTTTCCCAGAAAG-3′ (reverse); PSA, 5′-ATGTGGGTCCCGGTTGTCTTCCTCACCCTGTC-3′ (forward) and 5′-TCAGGGGTTGGCCACGATGGTGTCCTTGATC-3′ (reverse); and GAPDH, 5′-ACCACAGTCCATGCCATCA-3′ (forward) and 5′-TCCACCACCCTGTTGCTGT-3′ (reverse). The thermal profiles for β2M, AR, PSA, and GAPDH cDNA amplification were 25–30 cycles starting with denaturation for 1 min at 94 °C, followed by 1 min of annealing at 64 °C (for β2M), 61 °C (for AR), 55 °C (for PSA), and 60 °C (for GAPDH), and 1 min of extension at 72 °C. RT-PCR products were analyzed by 1.2% agarose gel electrophoresis. Quantity one-4.1.1 Gel Doc gel documentation software (Bio-Rad) was used for quantification of mRNA expression.

Western Blot Analysis—The cell lysates were prepared from prostate cancer cells as described previously (10). The concentration of protein was determined by the Bradford method using Coomassie Plus protein reagent (Pierce). Western blot analysis was performed by a Novex system (Invitrogen). Primary antibodies against human β2M, AR, PSA, SREBP-1, SREBP-2, FAS, MAPK/ERK (Santa Cruz Biotechnology), Akt, phospho-Akt (Ser^{473}), and phospho-p44/p42 MAPK (Thr^{202}/Tyr^{204}) (Cell Signaling Technology) were used. The corresponding secondary antibodies conjugated with horseradish peroxidase were purchased from GE Healthcare. Detection of protein bands was assayed by enhanced chemiluminescence Western blotting detection reagents (GE Healthcare).

Plasmid Construction—A luciferase reporter construct that contained the 5′-flanking region (−5400 to +580) of the full-length human AR (hAR) promoter was kindly provided by Dr. Donald J. Tindall (Mayo Clinic, Rochester, MN). The deletion constructs including, ΔA (deletion of −600 to −40), ΔB (deletion of −1100 to −600), and ΔC (deletion of −1600 to −1100) within the hAR promoter luciferase vector; and ΔEGR-1 binding site (5′-TCGCCCACGCTG-3′, −181 to −170), ΔSREBP-1 binding site (5′-CCTCGCCTCCAC-3′, −347 to −336), and ΔAP-1 binding site (5′-GCTTGGTCATG-3′, −475 to −465) within the hAR/SacI promoter (deletion of −4700 to −740) luciferase vector were generated by a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All of the plasmid construct DNA sequences were confirmed by DNA sequencing.

Transfection and Luciferase Activity Assay—LNCaP and C4-2B cells were plated at a density of 1.5×10^5 cells/well in 12-well plates 24 h before transfection. Plasmid DNAs were transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Each transfection

reaction contained 1.25 μg of tested DNA construct and 0.25 μg of a transfection efficiency control cytomegalovirus promoter β-galactosidase plasmid. After 6 h of incubation, DNA-liposome mixtures were replaced by fresh medium without fetal bovine serum. After overnight incubation, the transfected cells were treated with reagents or vehicles. After 24 h of additional incubation, the cells were harvested and lysed in 1× reporter lysis buffer (Promega). For luciferase activity assay, 20 μl of the lysate supernatant was mixed with 100 μl of the luciferase substrate (Promega) and detected by a luminometer (Monolight 3010 luminometer; PharMingen, San Diego, CA). For β-galactosidase activity assay, 100 μl of the supernatant was mixed with 100 μl of 2× β-galactosidase substrate (200 mM sodium phosphate buffer, pH 7.3, 2 mM $MgCl_2$, 100 mM β-mercaptoethanol and 1.33 mg/ml *o*-nitrophenyl-β-D-galactopyranoside) and incubated at 37 °C for 30 min. β-Galactosidase activity was detected by a microplate reader (model 680; Bio-Rad) at 405 nm wavelength. The data were presented as the normalized luciferase activity (the means ± S.D.) defined as the luciferase activity normalized to internal control β-galactosidase activity. All of the experiments were performed as three independent experiments with duplicate assays.

Electrophoretic Mobility Shift Assay (EMSA)—LNCaP and C4-2B cells were cultured in T-medium with 5% fetal bovine serum until 80% confluence. The cells were then switched to 1-day complete serum-free condition and then treated with β2M mAb (5 μg/ml), β2M mAb preincubated with β2M or control IgG for an additional 24 h. The nuclear extracts were prepared by a NucBuster™ protein extraction kit (Novagen, San Diego, CA). The specific double-stranded oligonucleotide of SREBP-1 binding site within hAR promoter region used as a probe was 5′-TTCCTCCCTC**CCTCGCCTCCAC**CCTGTT-GGTT-3′. The double-stranded oligonucleotide was end-labeled with [γ-^{32}P]ATP (3,000 Ci/mmol at 10 mCi/ml) using T4 polynucleotide kinase (New England BioLabs, Beverly, MA). Forty thousand cpm of the labeled probe and 5 μg of nuclear extracts were incubated with EMSA buffer containing 20 mM HEPES (pH 8.0), 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 20% glycerol, 500 ng of sonicated salmon sperm DNA, and 1 μg of poly(dI-dC) at 30 °C for 30 min. The samples were subjected to 6% nondenaturing DNA retardation gel and a Novex TBE system (Invitrogen). For the competition experiment, a ^{32}P-unlabeled oligonucleotide probe (100×) was preincubated with nuclear extracts for 30 min at room temperature before the addition of the ^{32}P-probe. After electrophoresis, the gels were dried with a Gel Dryer (model 583; Bio-Rad) and exposed to BioMax film (Kodak).

Chromatin Immunoprecipitation (ChIP) Assay—ChIP was performed by a ChIP-IT kit (Active Motif, Inc., Carlsbad, CA). Briefly, LNCaP and C4-2B cells were serum-starved for 24 h and then treated with or without β2M mAb (5 μg/ml) for an additional 24 h. The formaldehyde-fixed chromatins were prepared from these prostate cancer cells and were sheared into 200–1,500 bp of DNA fragments by enzymatic shearing mixture for 10 min at 37 °C. A portion of the fixed and sheared chromatin fragments was reversed and used as input DNA. The other chromatin DNA fragments were immunoprecipitated by anti-SREBP-1 antibody (Santa Cruz Biotechnology), and DNA was extracted and purified from the immunoprecipitate. A PCR primer pair to amplify the SREBP-1 binding site is 5′-TGG-CAGCCAGGAGCAGGTATT-3′ (forward) and 5′-TTTC-CTGGAGGCCAGCACTCAC-3′ (reverse). A negative PCR primer pair included in the ChIP-IT kit was used as a negative control. To further quantify the PCR products of ChIP, we conducted quantitative real time PCR. Each purified DNA sample was mixed with Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) and a primer pair of the SREBP-1 binding site (see above), and quantitative PCR was performed using an iCycler iQ real time PCR detection system (Bio-Rad) for two independent experiments with duplicate assays. The results were normalized by input (assigned as 1.0-fold without treating β2M mAb) for each cell line.

Immunohistochemical Staining—Primary anti-AR antibody purchased from Santa Cruz Biotechnology (1:100 dilution) was used. Tissue specimens were deparaffinized, rehydrated, and subjected to pressure-cooking antigen retrieval at 125 °C and 20 p.s.i. for 30 s, 10 min of double endogenous enzyme block, 4 °C for overnight primary antibody reaction, and 30 min of EnVision+ dual link and streptavidin-peroxidase system incubation. The signals were detected by adding substrate hydrogen peroxide using diaminobenzidine as the chromogen and counterstained with hematoxylin. The staining reagents were obtained from Dako Corporation (Carpinteria, CA).

Statistical Analysis—The statistical analyses were performed as described previously (35). Student's *t* test and two-tailed distribution were applied in the analysis of statistical significance.

RESULTS

Blockade of β2M Down-regulated AR and PSA Expression in Human Prostate Cancer Cells—In support of our previous report (7), we observed that β2M knockdown can be achieved efficiently by genetic manipulation using β2M siRNA (siβ2M) in a human prostate cancer cell line, C4-2B. We observed that mRNA levels of β2M, AR, and PSA were dramatically decreased in β2M knockdown C4-2B cells compared with parental (P) and control scramble siRNA (Scramble) C4-2B cells (Fig. 1*A*). In addition to decreased mRNA levels of β2M, AR, and PSA, endogenous proteins of secreted (from conditioned media) and soluble (from cell lysates) β2M, AR, and PSA were also greatly reduced in siβ2M C4-2B cells compared with P and Scramble C4-2B cells (Fig. 1*B*). Furthermore, these results were supported by an *in vivo* subcutaneous C4-2B xenograft mouse model (7) where immunohistochemical staining confirmed that AR expression was greatly decreased in siβ2M-treated C4-2B tumors compared with P and Scramble siRNA-treated C4-2B tumors (Fig. 1*C*). Mouse serum PSA levels were also markedly decreased in siβ2M-treated C4-2B tumors (1.03 ± 0.52 ng/ml, $n = 5$, after 28 days of treatment) compared with Scramble siRNA-treated C4-2B tumors (19.70 ± 9.04 ng/ml, $n = 5$). These *in vitro* and *in vivo* data suggested that blockade of intracellular β2M by β2M siRNA greatly inhibited the expression of AR and PSA mRNA and protein in prostate cancer cells.

To test whether interrupting β2M from extracellular sources may also affect AR and PSA expression as well as cell growth of prostate cancer cells, we employed a new β2M mAb to neutral-

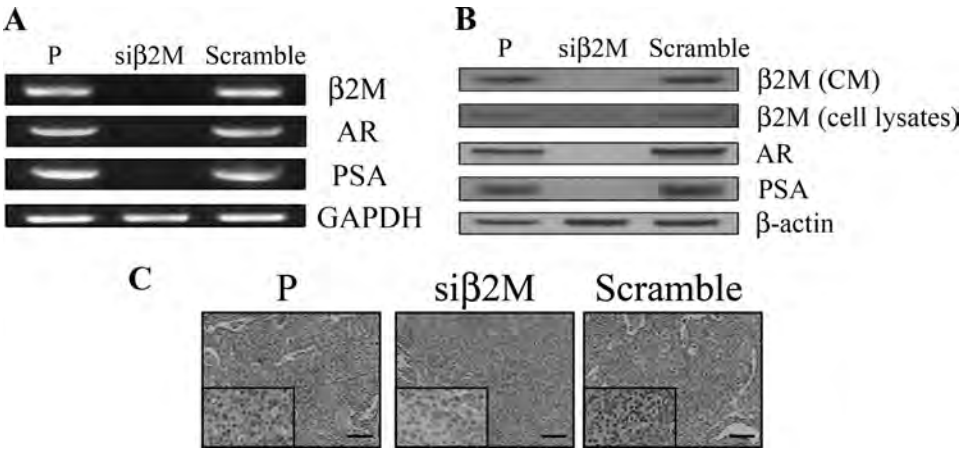

FIGURE 1. ***β2M siRNA inhibited expression of AR and PSA mRNA and protein in prostate cancer cells in vitro and in vivo.*** *A*, siβ2M dramatically decreased expression of β2M, AR, and PSA mRNA in C4-2B prostate cancer cells analyzed by RT-PCR. Expression of GAPDH was used as a loading control. *P*, parental nontransfected C4-2B cells; *Scramble*, control scramble siRNA transfected C4-2B cells. *B*, siβ2M also markedly inhibited expression of secreted β2M (from conditioned media, *CM*), soluble β2M (from cell lysates), AR, and PSA protein in siβ2M C4-2B cells compared with P and Scramble C4-2B cells assayed by Western blot. β-Actin was used as an internal loading control. *C*, immunohistochemical staining analysis of AR in P (parental, untreated), siβ2M (β2M siRNA-treated), and Scramble (control scramble siRNA-treated) C4-2B tumor specimens from subcutaneous mouse xenografts (7). This result revealed that siβ2M greatly inhibited AR expression in C4-2B subcutaneous tumors *in vivo*. *Scale bars*, 100 μm.

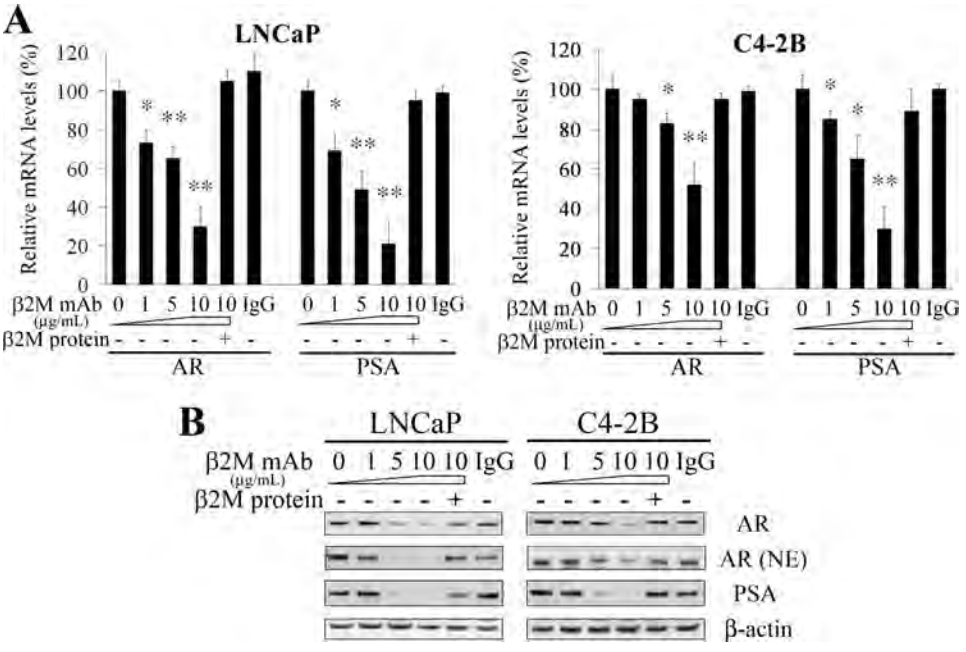

FIGURE 2. ***β2M mAb decreased expression of AR and PSA in prostate cancer cells.*** *A*, β2M mAb decreased the steady-state mRNA levels of AR and PSA in a dose-dependent manner (0–10 μg/ml) in LNCaP and C4-2B AR-positive prostate cancer cells determined by semi-quantitative RT-PCR. The inhibitory effect was restored by preincubation of β2M mAb with purified β2M protein. Isotype control IgG (10 μg/ml) did not significantly affect AR and PSA mRNA expression. The relative mRNA levels of AR and PSA, normalized by GAPDH mRNA, were measured by Gel Doc gel documentation software (Bio-Rad). The relative mRNA levels (%) were assigned as 100% in the absence of β2M mAb treatment. *, $p < 0.05$; **, $p < 0.005$, significant differences from the β2M mAb-untreated group. The data represent the means ± S.D. of independent triplicate experiments. *B*, β2M mAb also inhibited total AR, nuclear AR (*NE*, nuclear extracts) and PSA protein expression in a dose-dependent pattern (0–10 μg/ml) in LNCaP and C4-2B cells assayed by Western blot. The inhibitory effect was abrogated by preincubation of β2M mAb with β2M protein. Control IgG (10 μg/ml) did not change AR and PSA protein expression. β-Actin was used as an internal loading control.

ize extracellular β2M in an attempt to interrupt β2M downstream signaling. As shown in Figs. 2, β2M mAb (0 to 10 μg/ml) significantly decreased both steady-state mRNA levels and protein amounts of AR and PSA in LNCaP and C4-2B cells in a dose-dependent pattern determined by semi-quantitative RT-PCR and Western blot. Considering the specificity of the inhibitory effect of β2M mAb, purified β2M protein could rescue AR and PSA inhibition by β2M mAb in prostate cancer cells. Control IgG did not affect AR and PSA expression. In addition to decreasing endogenous total AR protein, β2M mAb also inhibited nuclear AR levels in LNCaP and C4-2B cells (Fig. 2*B*). These data suggested that antagonizing extracellular β2M by β2M mAb reduced AR and PSA transcription and translation in prostate cancer cells.

β2M mAb Blocked AR Transcription via Down-regulation of Sterol Regulatory Element-binding Protein-1 Activity—AR gene transcription was studied in LNCaP and C4-2B cells by transfecting these cells with either the full-length or the deletion constructs of the 5′-flanking region of hAR promoter luciferase reporters (*i.e.* from −5400 to +580). Consistent with previous RT-PCR and Western blot results (Fig. 2), β2M mAb (0–10 μg/ml) significantly decreased the full-length hAR promoter luciferase activity in a concentration-dependent pattern (Fig. 3*A*), and purified β2M protein was shown to restore the inhibition. Additionally, isotype-matched control IgG failed to decrease the hAR promoter luciferase activity in LNCaP and C4-2B cells. To further identify the responsible *cis*-acting element in the hAR promoter region, we conducted a hAR promoter deletion study. Three deletion constructs of hAR promoter fragment (ΔA, ΔB, and ΔC; Fig. 3*B*) were generated and confirmed the DNA sequence. After transfection into LNCaP and C4-2B cells, β2M mAb significantly inhibited the activities of the full-length hAR, ΔB (deletion of −1100 to −600), and ΔC (deletion of −1600 to −1100) promoter luciferase constructs (Fig. 3*B*). However, β2M mAb did not affect ΔA promoter luciferase activity (deletion of −600 to −40; Fig. 3*B*). Among the three deletion constructs, decreased basal AR promoter luciferase activity was observed only in the ΔA construct when tested in LNCaP and C4-2B cells. Control IgG did not significantly change the promoter activities of

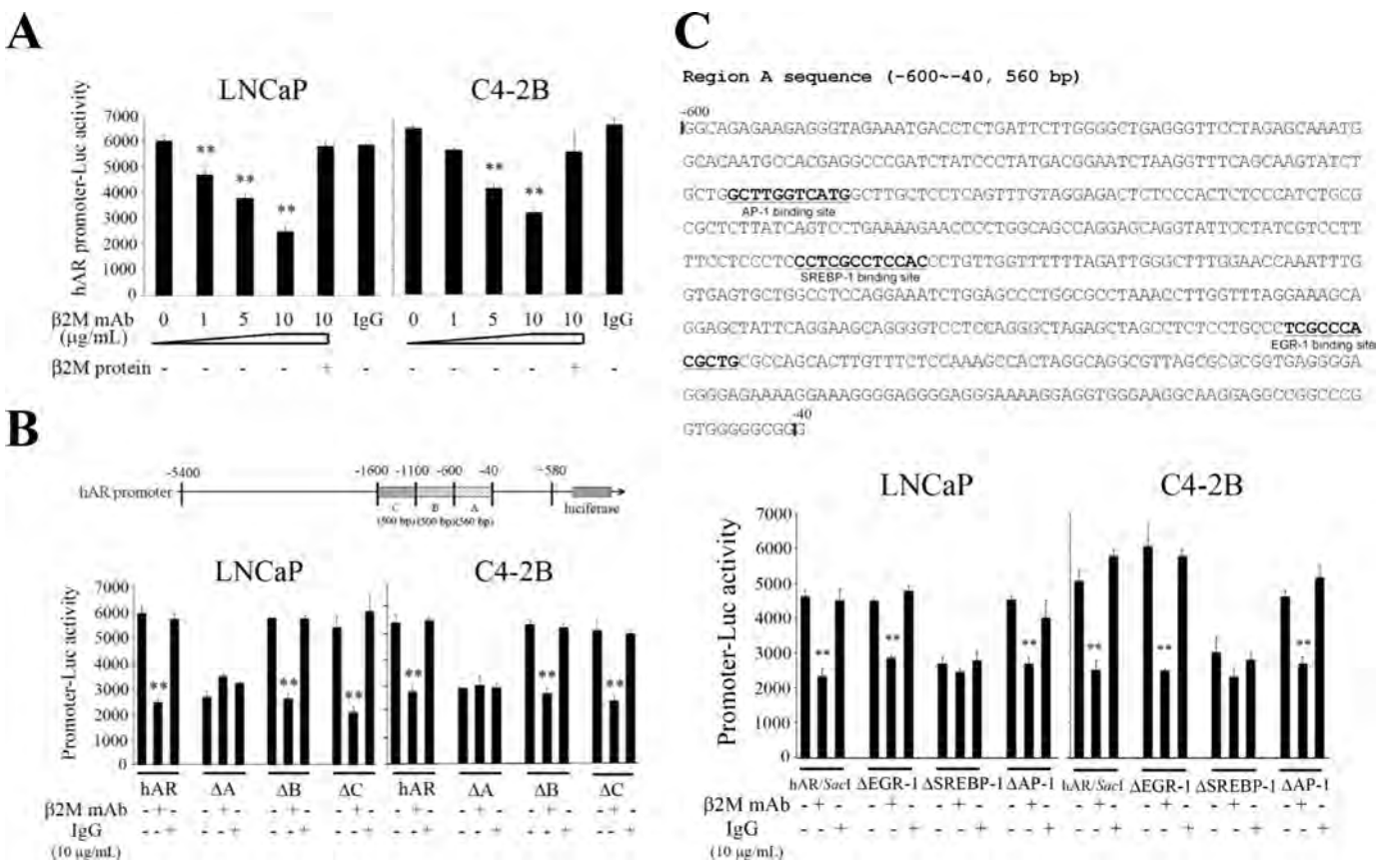

FIGURE 3. **SREBP-1 binding site within the 5′-flanking promoter region of hAR gene is responsible for AR transcriptional activity mediated by β2M mAb.** *A*, β2M mAb decreased the full-length hAR promoter (−5400 to +580) luciferase activity with a concentration-dependent pattern (0–10 μg/ml) in LNCaP and C4-2B cells. Purified β2M protein restored the inhibitory effect of hAR promoter activity regulated by β2M mAb. Control IgG did not suppress hAR promoter reporter activity. *B*, region A (−600 to −40) is responsible for hAR promoter luciferase activity mediated by β2M mAb in LNCaP and C4-2B cells. β2M mAb (10 μg/ml) significantly decreased the promoter luciferase activities of the deleted region B (ΔB, −1100 to −600) and C (ΔC, −1600 to −1100) in hAR promoter report constructs but did not affect the luciferase activity of the ΔA (−600 to −40) construct. Isotype control IgG (10 μg/ml) did not significantly change the promoter reporter activities of all deletion constructs. *C*, the DNA sequence of region A (560 bp) contains an EGR-1 binding site (−181 to −170), a SREBP-1 binding site (−347 to −336), and an AP-1 binding site (−475 to −465). Among the three deletion constructs (ΔEGR-1, ΔSREBP-1, and ΔAP-1 binding sites), the promoter luciferase activities of ΔEGR-1 and ΔAP-1 binding site constructs were significantly inhibited by β2M mAb in LNCaP and C4-2B cells. Only a slight decrease of promoter luciferase activity was observed in a ΔSREBP-1 binding site construct treated with β2M mAb in prostate cancer cells. Control IgG did not change the promoter reporter activities of these three deletion constructs. All of the promoter luciferase activity data (in Fig. 3) were normalized by internal control β-galactosidase activity and expressed as the means ± S.D. of three independent duplicate experiments. **, $p < 0.005$.

all these vector constructs. These results suggested that a potential *cis*-acting element mediating AR transcriptional activity by β2M mAb may reside at region A. Because the full-length hAR promoter reporter construct is ~6 kb in length (from −5400 to +580), we further used a restriction enzyme, SacI, to generate a shorter promoter luciferase construct, a hAR/SacI vector (2 kb only, deletion of −4700 to −740), and tested this new reporter vector activity in LNCaP and C4-2B cells exposed to either β2M mAb or IgG. The basal luciferase activity of the truncated hAR/SacI promoter vector decreased slightly when compared with the full-length hAR promoter activity assayed in LNCaP and C4-2B cells (Fig. 3, *B* and *C*). These results indicated that the *cis*-elements spanning from −4700 to −740 of the hAR promoter were not responsible for AR transcriptional regulation in human prostate cancer cells.

To determine the precise *cis*-acting elements in region A of the AR promoter responsible for β2M mAb-mediated regulation, we searched the data base and predicted three potential *cis*-acting elements in this region: the EGR-1 binding site (−181 to −170), SREBP-1 binding site (−347 to −336), and AP-1 binding site (−475 to −465) (Fig. 3*C*). Subsequently, we generated three respective deletion constructs and tested their luciferase reporter activities in prostate cancer cells exposed to either β2M mAb or control IgG. In resemblance to the truncated hAR/SacI luciferase construct, we found that β2M mAb inhibited ΔEGR-1 and ΔAP-1 but not ΔSREBP-1 binding site deletion construct activities in LNCaP and C4-2B cells (Fig. 3*C*). Decreased basal promoter luciferase activity was noted in the ΔSREBP-1 binding site deletion construct (Fig. 3*C*). Control IgG did not significantly change the promoter reporter activities of all deletion constructs. These data, taken together, demonstrated that the SREBP-1 binding site located within the 5′-flanking hAR promoter region is important for hAR promoter activity regulated by β2M mAb in AR-positive human prostate cancer cells.

Confirmation of Nuclear SREBP-1 Protein Interaction with the SREBP-1 Binding Site within the hAR Promoter by EMSA and ChIP Assay—We conducted EMSA to further validate whether the SREBP-1 transcription factor is a key protein regulating AR transcriptional activity through β2M mAb in prostate cancer cells. As shown in Fig. 4*A*, nuclear extracts prepared

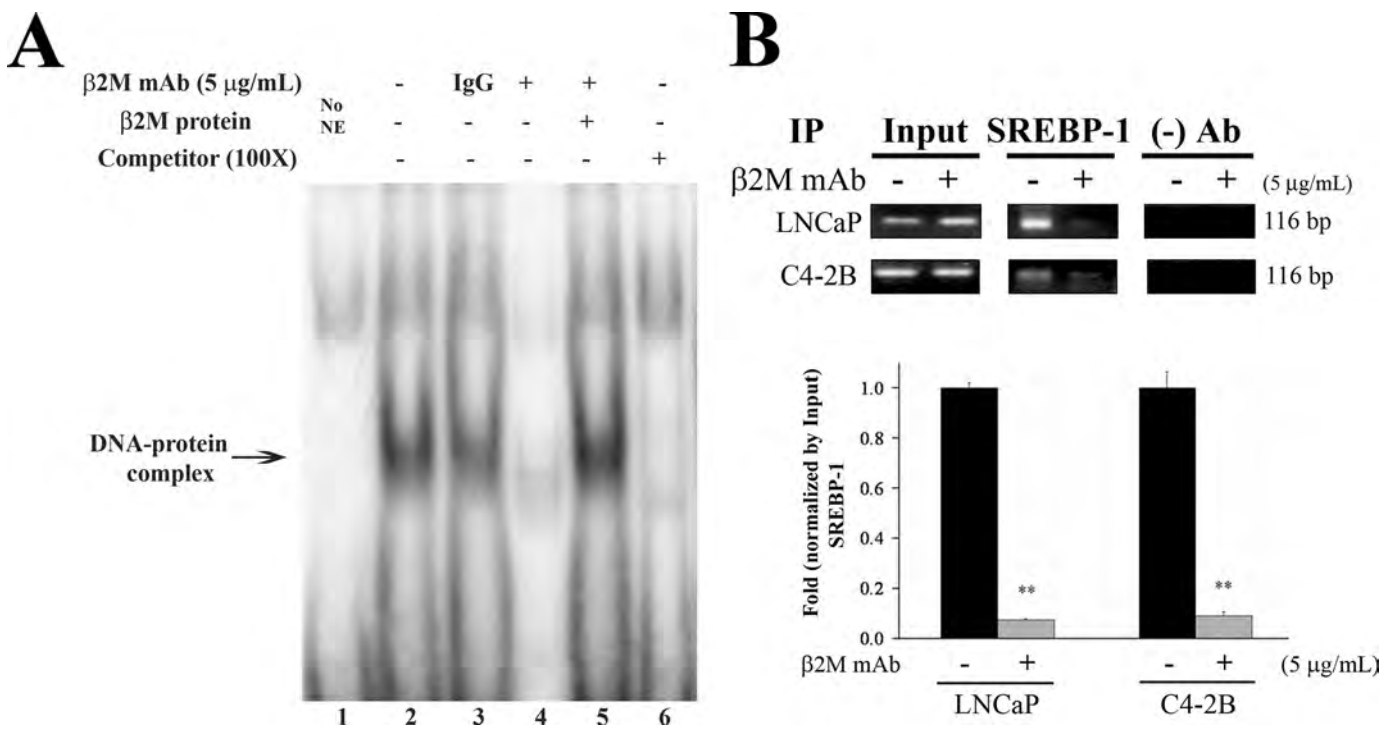

FIGURE 4. **β2M mAb inhibited the interaction between SREBP-1 and its binding *cis*-acting element located in the 5′-flanking hAR promoter region in prostate cancer cells.** *A*, EMSA. LNCaP cells were exposed to β2M mAb, control IgG (5 μg/ml), or vehicle for 24 h in serum-free conditions. The cells were harvested, and nuclear extracts were prepared. EMSA was performed by incubating nuclear extracts with the ^{32}P-labeled SREBP-1 oligo-DNA probe and Novex TBE system (Invitrogen). *Lane 1*, no nuclear extracts (*NE*) added. *Lanes 2* and *3*, nuclear SREBP-1-oligo-DNA complex, not affected by control IgG. *Lane 4*, β2M mAb greatly inhibited this nuclear protein-DNA complex formation. *Lane 5*, purified β2M protein could rescue the inhibitory binding effect of β2M mAb. *Lane 6*, this complex was competed off by adding 100-fold ^{32}P-unlabeled specific SREBP-1 oligo-DNA probe. *B*, ChIP assay. LNCaP and C4-2B cells were treated with or without β2M mAb (5 μg/ml) for 24 h. The chromatin and nuclear proteins were crossed-linked by formaldehyde and sheared by enzymatic shearing mixture (ChIP-IT kit, Active Motif, Inc.) and subjected to immunoprecipitation (*IP*) assay by anti-SREBP-1 antibody or without antibody as a negative control. In the PCR product from chromatin DNA fragments immunoprecipitated by anti-SREBP-1 antibody as templates, a predicted single DNA band (116 bp, *top panel*) was amplified and visualized in LNCaP and C4-2B cells. This PCR product was decreased by treatment with β2M mAb in prostate cancer cells. In addition, quantitative real time PCR of ChIP was performed (*bottom panel*). The results of quantitative PCR showed that β2M mAb significantly down-regulated the interaction between SREBP-1 and the SREBP-1 binding site in the 5′-flanking hAR promoter, with a 13.3 and 11.0-fold decrease in LNCaP and C4-2B cells, respectively. The quantitative PCR data were normalized by input and assigned as 1.0-fold without β2M mAb for each prostate cancer cell line. **, $p < 0.005$.

from β2M mAb-treated LNCaP cells showed a greatly decreased the ^{32}P-oligo-DNA and SREBP-1 binding complex (*lane 4*) compared with the DNA-nuclear protein complex without β2M mAb or control IgG treatment in LNCaP cells (*lanes 2* and 3). Purified β2M protein was shown to abrogate the complex formation decreased by β2M mAb exposure (*lane 5*). The specificity of the binding of ^{32}P-oligo-DNA probe with nuclear SREBP-1 protein in LNCaP cells was shown by the effective competition of ^{32}P unlabeled oligo-DNA for this binding complex (*lane 6*).

We performed ChIP to investigate whether the interaction between nuclear SREBP-1 and its binding *cis*-acting element is affected by β2M mAb in the chromatin environment in prostate cancer cells. An expected single DNA band (116 bp) was detected by a PCR primer set to amplify a SREBP-1 binding region within hAR promoter in LNCaP and C4-2B cells, whereas the chromatin DNA fragments immunoprecipitated by anti-SREBP-1 antibody were used as templates (Fig. 4*B*, *top panel*). The levels of this amplified PCR product were decreased by exposure to β2M mAb in prostate cancer cells (Fig. 4*B*). In addition, utilizing real time quantitative PCR as a readout, we observed that β2M mAb caused significant reduction of the interaction between SREBP-1 and its binding site within the AR promoter region, with 13.3- and 11.0-fold decreases in LNCaP and C4-2B cells, respectively (Fig. 4*B*, *bottom panel*). In summary, EMSA and ChIP data confirmed that β2M mAb inhibited the interaction between nuclear SREBP-1 and its *cis*-acting element within the AR promoter region, which accounts for the down-regulated AR transcriptional activity in prostate cancer cells.

A Triad Relationship among β2M, SREBP-1, and AR Is Involved in the Regulation of Fatty Acid Levels and Cell Viability in Prostate Cancer Cells—We evaluated the potential triad relationship among the expression of β2M, SREBP-1, and AR in prostate cancer cells. As shown in Fig. 5*A*, β2M mAb (0 to 10 μg/ml) specifically inhibited expression of precursor (125 kDa) and mature nuclear (68 kDa) SREBP-1 proteins in a concentration-dependent manner but did not affect expression of SREBP-2, which is a SREBP-1 isoform. Purified β2M protein rescued the inhibitory effect of endogenous SREBP-1 expression by β2M mAb. Control IgG did not affect SREBP-1 and SREBP-2 expression. A fatty acid biosynthetic oncogene, FAS, which is a downstream target gene of SREBP-1, was also shown to be decreased by β2M mAb in a dose-dependent inhibition (Fig. 5*A*). In addition, we observed that β2M mAb (5 μg/ml, 24 h treatment) significantly decreased fatty acid levels in LNCaP (20.8 ± 2.9%) and C4-2B (26.6 ± 2.1%) cells. To investigate the role of SREBP-1 in regulating AR expression, we conducted functional

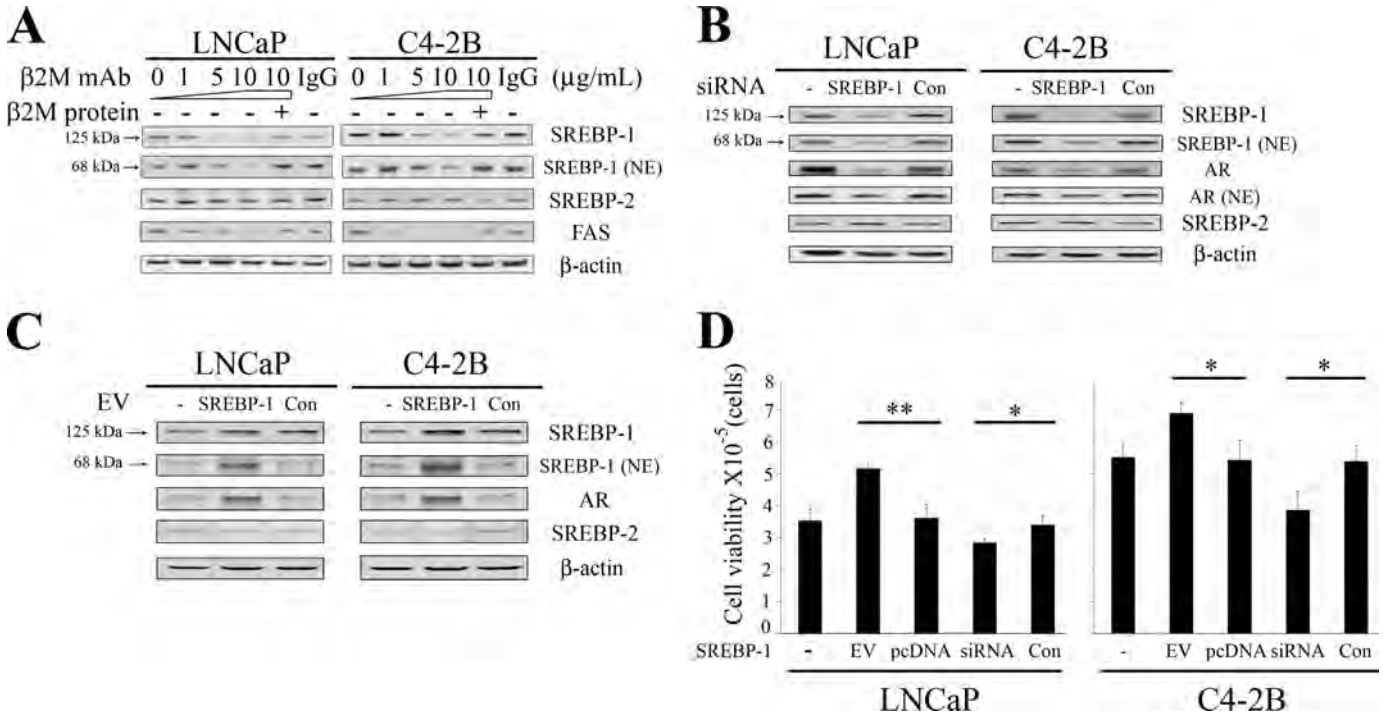

FIGURE 5. **A triad relationship among β2M, SREBP-1, and AR expression in prostate cancer cells.** *A*, β2M mAb decreased the expression of precursor SREBP-1 (125 kDa), mature nuclear SREBP-1 (68 kDa), and FAS in a concentration-dependent pattern (0–10 μg/ml) in LNCaP and C4-2B cells as determined by Western blot. The inhibitory effect of β2M mAb on endogenous SREBP-1 and FAS expression was restored by preincubation of β2M mAb with purified β2M protein. Isotype control IgG (10 μg/ml) did not affect expression of these proteins. β2M mAb did not change the expression of SREBP-2, which is an isoform of SREBP-1. β-Actin was used as a loading control. *B*, a sequence-specific siRNA of SREBP-1 decreased the expression of precursor and nuclear SREBP-1 proteins in LNCaP and C4-2B cells. Because of down-regulation of SREBP-1 by SREBP-1 siRNA, total AR, and nuclear AR (NE) expression was also inhibited in LNCaP and C4-2B cells. To test the specificity of SREBP-1 siRNA, SREBP-2 expression was not changed by this siRNA. Control nonspecific siRNA (*Con*) did not affect SREBP-1, AR, and SREBP-2 expression. *C*, overexpressing SREBP-1 by a SREBP-1 expression vector (*EV*) increased the expression of precursor and nuclear SREBP-1 as well as endogenous AR protein in LNCaP and C4-2B cells. SREBP-1 EV did not affect SREBP-2 expression. *Con*, control empty expression vector. *D*, overexpressing or knocking down SREBP-1 by SREBP-1 EV or SREBP-1 siRNA significantly increased or decreased LNCaP and C4-2B cell viability after a 4-day transfection. Empty expression vector (pcDNA 3.1; Invitrogen) or nonspecific siRNA (*Con*) were used as control groups. The cell numbers (cell viability) were measured by a hemacytometer (Hausser Scientific, Horsham, PA) and crystal violet staining method. *, $p < 0.05$; **, $p < 0.005$, significant differences from the control groups. The data represent the means ± S.D. of two independent experiments replicated three times.

studies to knock down and overexpress SREBP-1 in prostate cancer cells. A sequence-specific siRNA of SREBP-1 caused a marked decrease of both precursor and mature nuclear SREBP-1 proteins in LNCaP and C4-2B cells (Fig. 5*B*). Down-regulation of SREBP-1 by SREBP-1 siRNA also inhibited the expression of total AR and nuclear AR proteins in LNCaP and C4-2B cells (Fig. 5*B*). In testing the specificity of SREBP-1 siRNA, SREBP-2 expression was shown not to be affected by this siRNA. Control nonspecific siRNA did not inhibit expression of SREBP-1, SREBP-2, and AR. Conversely, overexpressing SREBP-1 by a SREBP-1 expression vector increased expression of precursor and nuclear SREBP-1 as well as AR but not SREBP-2 in LNCaP and C4-2B cells (Fig. 5*C*). In addition, overexpressing or knocking down SREBP-1 significantly increased or decreased cell viability (Fig. 5*D*) and fatty acid levels (data not shown) in prostate cancer cells. These data are consistent with previous reports and in aggregate reveal that β2M is a pleiotropic signaling molecule (6, 7, 36) and has a triad relationship with SREBP-1 and AR, which determines the growth and survival of prostate cancer cells. β2M-mediated signaling is important for the maintenance of SREBP-1 expression and SREBP-1 regulates AR expression and prostate cancer cell growth and survival (Fig. 5, *B–D*). Likewise, a reciprocal relationship has been reported between AR and β2M in which androgens and AR regulated β2M expression (8) and β2M mediated AR expression and prostate cancer cell growth and survival (Figs. 1 and 2) (10).

The Involvement of the MAPK/ERK Signaling Pathway in β2M mAb Inhibition of SREBP-1 and AR Expression in Prostate Cancer Cells—To determine the signaling mechanism by which β2M mAb inhibited SREBP-1 and AR expression in prostate cancer cells, we examined the β2M mAb-mediated status of MAPK/ERK and PI3K/Akt signaling pathways, which have been reported to regulate SREBP-1 and AR expression (25, 37, 38). The expression levels of both the phosphorylated and total proteins of Akt and MAPK were analyzed by Western blot. As shown in Fig. 6*A*, β2M mAb decreased expression of phospho-Akt (Ser^{473}) and phospho-MAPK (Thr^{202}/Tyr^{204}) proteins in LNCaP and C4-2B cells within 2 h in a time-dependent pattern. However, β2M mAb did not suppress total Akt and MAPK expression. We also observed that AR and SREBP-1 proteins were not decreased by β2M mAb within 2 h of treatment (Fig. 6*A*). To further confirm the responsible signaling pathways regulating SREBP-1 and AR expression, we evaluated the effect of U0126 (a MAPK selective inhibitor), LY294002 (a PI3K selective inhibitor), and H-89 (a PKA selective inhibitor) on prostate cancer cells. β2M mAb and U0126 greatly inhibited expression of SREBP-1 and AR in LNCaP cells (Fig. 6*B*). FAS was also

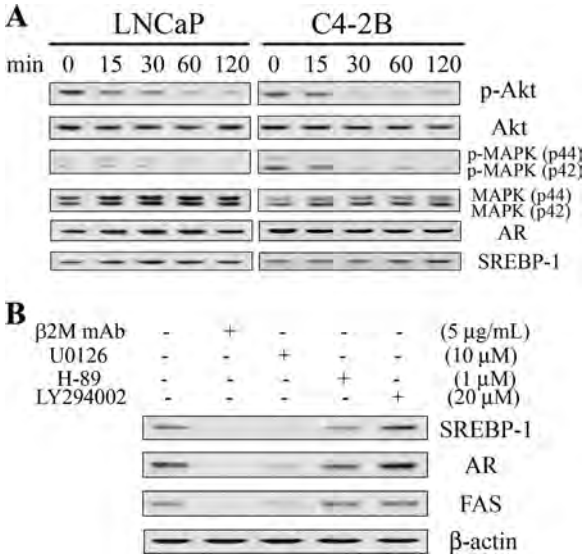

FIGURE 6. **β2M mAb inhibited SREBP-1 and AR expression through a MAPK/ERK signaling pathway in prostate cancer cells.** *A*, β2M mAb (10 μg/ml) decreased phospho-Akt (p-Akt, Ser^{473}) and phospho-MAPK (p-MAPK, Thr^{202}/Tyr^{204}) protein expression but did not inhibit total Akt and MAPK (p44/p42) protein expression in LNCaP and C4-2B cells at different time points (0, 15, 30, 60, and 120 min) as assayed by Western blot. AR and SREBP-1 protein expression was not affected by β2M mAb within 2 h of treatment. *B*, β2M mAb (5 μg/ml) and U0126 (10 μM, a MAPK selective inhibitor) greatly inhibited expression of endogenous SREBP-1, AR, and FAS proteins in LNCaP cells with 24 h of treatment as determined by Western blot. H-89 (1 μM, a PKA selective inhibitor) slightly decreased SREBP-1, AR, and FAS expression. However, LY294002 (20 μM, a PI3K selective inhibitor) appeared to increase SREBP-1 and AR expression. β-Actin was used as an internal loading control.

decreased by U0126 treatment. H-89 slightly decreased the expression of these proteins. However, LY294002 appeared to enhance SREBP-1 and AR expression in LNCaP cells. Interrupting the PI3K/Akt signaling pathway with LY294002 increased AR, and PSA protein expression in LNCaP cells has been reported (25). Similar results were observed when C4-2B cells were treated with these signaling pathway inhibitors (data not shown). These data suggested that a MAPK/ERK signaling pathway may play a dominant role in the regulation of SREBP-1 and AR expression through β2M mAb in prostate cancer cells.

DISCUSSION

This study investigated the pleiotropic signaling functions of β2M, which confer growth, survival, and metastasis benefits to prostate cancer cells. We focus here on the critical role of β2M in the regulation of AR through SREBP-1, which plays a key role in lipid homeostasis, regulating its downstream target genes, such as FAS, and accumulation of fatty acids and lipid droplets (supplemental Fig. S1) by Oil Red O staining (39) in prostate cancer cells. We identified a triad relationship among β2M, SREBP-1, and AR. In response to the β2M-mediated cell signaling, SREBP-1 regulated AR expression by altering AR gene transcription. Conversely, androgens and AR were also found to mediate SREBP-1 expression reciprocally in androgen-responsive prostate cancer cells (40, 41). These data, taken together with previous reports from our laboratory and others that documented the regulatory role of β2M on AR expression (10) and also androgens and AR regulation of β2M expression (8), support the triad relationship among β2M, SREBP-1, and AR.

There are several important clinical implications of this triad relationship. 1) β2M could be an important driver modulating SREBP-1 and AR expression in prostate cancer cells. It has been shown that upon androgen-refractory progression of human prostate cancer, dysregulated expression of β2M (2, 7, 42), SREBP-1 (34), and AR (43, 44) are observed. By employing β2M mAb as a therapeutic agent, we and others found that this antibody caused massive cell death in human prostate and renal cancers (10, 11), multiple myeloma, leukemia, and lymphoma (9) without affecting the growth of normal cells. We propose that the inhibitory action of β2M mAb could act via inhibition of SREBP-1 expression, which is linked to FAS expression and lipogenic pathways that are known to regulate cell membrane integrity, energy metabolism, lipid raft-mediated signaling in cancer cells (27, 29, 45, 46), and AR expression, which is regarded as a growth and survival factor for human prostate cancer cells (43). 2) The pleiotropic cell signaling network activated by β2M could have a mediatory action on lipid metabolism and storage and lipid raft-directed cell signaling pathways. In this study, we showed that β2M mAb inhibited a large number of cell signaling networks, including MAPK, SREBP-1, AR, and PI3K/Akt. It is conceivable that these signaling networks are interconnected through lipid raft complexes (47, 48). The inhibitory action exerted by β2M mAb could affect the lipid composition of the raft structures, hence altering domain interactions and how downstream cell signal networks can be assembled and interact in a coordinated manner (45, 49). 3) β2M-regulated downstream signaling is highly dynamic and could affect the function of cells without AR expression. β2M mAb was shown to block the growth and downstream signaling of cancer but not normal cells regardless of their endogenous levels of AR (10), implying that AR function is not obligatory for the triad relationship. These observations also suggest that AR function could be bypassed by other redundant cell signaling networks mediated by soluble factors such as insulin-like growth factor I, epidermal growth factor, keratinocyte growth factor, and interleukin-6 (19, 20, 50, 51). Additional studies may be warranted to define how the triad relationship would function in normal *versus* cancer cells and in clinical prostate cancer, which characteristically contains cells with heterogeneous arrays of AR, including AR gene amplification and mutation and AR protein overexpression and silencing (52–54). In addition, the data presented in this communication are collected from the study of established AR-positive human prostate cancer cell lines, and further investigation of this concept in primary prostate cancer cells might be of importance.

The precursors of SREBP family proteins are endoplasmic reticulum membrane-anchored with the mature amino-terminal forms being translocated to the cell nucleus responsible for the activation of their target genes (27). Blockade of SREBP-1, a crucial transcriptional regulator for fatty acid and lipid biosynthesis, by β2M mAb inhibited expression of both precursor and mature nuclear SREBP-1 and FAS (Fig. 5*A*) in prostate cancer cells and interrupted cell growth and promoted apoptosis (10). We also observed that β2M mAb significantly decreased fatty acid contents and lipid droplet accumulation in LNCaP and C4-2B cells. However, the signaling mechanism regulating SREBP-1 and its downstream target gene expression by β2M

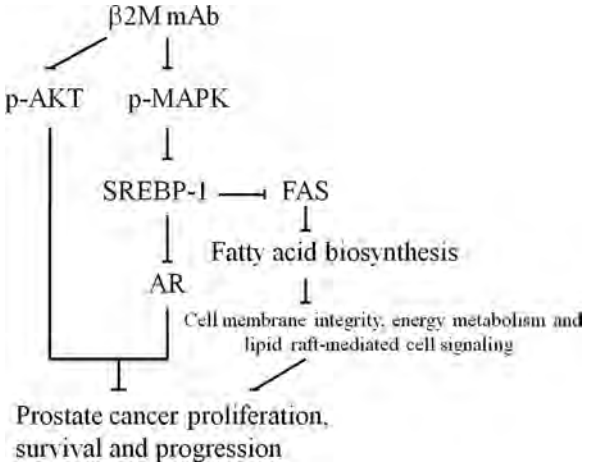

FIGURE 7. **Proposed mechanism for β2M mAb inhibition of AR and cell proliferation, survival, and progression through a MAPK/SREBP-1 signaling pathway in prostate cancer cells.** β2M mAb inhibited p-MAPK and p-AKT expression and decreased SREBP-1 expression in LNCaP and C4-2B cells. By the addition of U0126, a MAPK selective inhibitor, we observed down-regulation of SREBP-1 expression (see Fig. 6*B*). In addition, β2M mAb inhibited AR and FAS expression; the latter is known to regulate fatty acid biosynthesis, cell membrane integrity, energy metabolism, and lipid raft-regulated cell signaling (31, 32, 46), which ultimately control prostate cancer cell proliferation, survival, and progression.

mAb in prostate cancer is still unclear. Reports in the literature suggest that SREBP-1 is induced by PI3K/Akt and/or MAPK signaling pathways in liver cells, macrophages, and mammary epithelial and breast cancer cells (38, 55, 56). The inhibitors of MAPK and PI3K signaling pathways were demonstrated to down-regulate SREBP-1 and FAS expression and inhibit fatty acid synthesis in MCF-7 and HCT166 carcinoma cells (38). In LNCaP and C4-2B prostate cancer cells, we observed that U0126 (a MAPK selective inhibitor) inhibited SREBP-1, FAS, and AR protein expression (Fig. 6*B*), similar to the action of β2M mAb. However, LY294002 (a PI3K selective inhibitor) increased SREBP-1 and AR protein expression in AR-positive prostate cancer cells. Blocking the PI3K/Akt signaling pathway by LY294002 in LNCaP cells resulted in induced AR expression through activation of a Forkhead transcription factor, FOXO3a (25). H-89, a PKA selective inhibitor, did not significantly affect SREBP-1 expression (Fig. 6*B*). In addition, we showed that β2M mAb decreased p-MAPK protein expression in LNCaP and C4-2B cells (Fig. 6*A*), and this inhibition coincides with decreased SREBP-1 and AR expression (Figs. 2 and 5*A*). Our data collectively suggest that MAPK signaling is the dominant pathway that positively controls SREBP-1, AR, and FAS expression in AR-positive prostate cancer cells and that this cell signaling network may be different in cancer and normal cells.

In conclusion, these results show for the first time that: 1) interrupting intracellular or extracellular β2M using sequence-specific siRNA or mAb resulted in decreased expression of AR and PSA at the transcriptional and translational levels in AR-positive prostate cancer cells; 2) β2M mAb inhibited AR expression by blocking a MAPK signaling pathway, decreasing the expression of precursor and nuclear SREBP-1 and reducing the binding between SREBP-1 and its *cis*-element binding site in the hAR promoter region in LNCaP and C4-2B cells; 3) β2M mAb decreased fatty acid and lipid accumulation by inhibition of SREBP-1 and FAS expression and may disrupt cell membrane integrity, intracellular lipid raft-mediated cell signaling and energy metabolism; and 4) functional studies of SREBP-1 demonstrated that SREBP-1 regulated endogenous AR expression and cell viability in LNCaP and C4-2B cells. Collectively, these results suggest that β2M mAb is a novel therapeutic antibody capable of inhibiting the pleiotropic cell signaling network converging on β2M in prostate cancer cells (Fig. 7). This study also expands our understanding of the key regulatory role of β2M, which controls intracellular signaling pathways through the regulation of transcription factors such as cAMP-responsive element-binding protein (7, 57), hypoxia inducible factor1-α (HIF1-α) (57), and SREBP-1.

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